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Modifying current laboratory methods for rapid determination of colostral IgG concentration and colostral IgG absorption in the neonate

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Modifying current laboratory methods for rapid determination of colostral IgG concentration and colostral IgG absorption in the neonate

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CHAPTER ONE

GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized into eight chapters. The first chapter includes the dissertation organization and a literature review that details colostrogenesis, colostrum composition, neonatal development of the immune system and gastrointestinal tract, methods to measure colostrum quality, and methods to determine immunoglobulin transport in neonates. Chapter two details the development of the caprylic acid test to determined colostral IgG concentration. Chapter three discusses the on-farm adaptation and evaluation of the caprylic acid test and whole refractomtry and variables (parity, breed, storage, nutrient composition and bacterial contamination) that may potentially impact its accuracy at estimating IgG concentration. Chapter four looks at the impact of freeze/thaw cycles on the accuracy of the caprylic acid test and refractometry of whole colostrum as methods to determine IgG concentration in bovine colostrum. Chapter five characterizes the nutrient composition, bacterial contamination and IgG concentration of bovine colostrum that is available on U.S. dairy farms to potentially be fed to newborn calves. This chapter also highlights the percentage of colostrum samples that meet current industry recommendations for IgG concentration and bacterial contamination. Chapter six looks at the adaptation of the caprylic acid test to bovine serum as a method to determine failure of passive transfer in neonatal calves. Chapter seven explores the use of the Ussing chamber to determine IgG transport across the intestinal epithelium of neonatal mid-jejunum intestinal samples. Chapter eight provides an overall conclusion for each of the research projects completed, as well as recommendations for future research.

Literature Review

Introduction:

Newborn animals of many species rely on maternal colostrum **(MC)** to provide them with the nutrients needed to sustain life. Because immunoglobulins **(Ig)** cannot cross the placental structure of cattle, calves are born aggamaglobulinemic with little to no measurable circulating IgG or IgM. High quality MC fed within the first hours of life provides the calf with sufficient amounts of Ig to provide passive immunity for the first 30 – 90 days of life (Guy et al., 1994) until the immune system of the calf is better equipped to respond to pathogens on its own. Colostrum also provides the calf with nutrients necessary to sustain life. Enhancing passive transfer of colostral antibodies will lead to a decrease in calf mortality and an increase in producer profitability.

Achieving early and adequate intake of high quality MC is the single most important management practice influencing calf health and survival. Colostrum components exert effects on the gastrointestinal tract **(GIT)**, produce transient systemic metabolic and endocrine changes and have long lasting effects on immunoprotection as well as nutritional status of the newborn calf (Bösze, 2008). Colostrum provides the neonate with not only nutrients needed for survival, but also antibodies and maternal cells that are important to help defend against pathogens during the first weeks of life when the immune system is still developing.

Colostrum quality is related to IgG concentration in the MC. Currently producers can use a colostrometer to estimate MC quality. However, the colostrometer uses specific gravity for quality calibration (Fleenor and Stott, 1980) and readings are affected by temperature of MC as well as total solids **(TS)** content (Morin et al., 2000; Mechor et al., 1992). Using the colostrometer on MC that is cold (< 80 ^{o}F) will lead to readings that overestimate the actual IgG concentration of the sample. Likewise, samples that are too warm will have readings that underestimate the actual IgG concentration. Creating a more accurate cow-side test to determine MC quality will benefit dairy producers.as it will enhance their ability to make management decision to ensure that calves are receiving adequate IgG to achieve adequate passive transfer.

Current research methods to evaluate antibody transfer involves the use of newborn calves in a controlled situation in which the animals are not allowed to suckle from the dam and are fed a uniform MC or colostrum replacerment **(CR)** product within a set time after birth. Blood samples must be taken from the calf at defined intervals for the first 24 to 48 h of life. These larger field trials can unintentionally introduce confounding factors including ambient temperature, method of feeding, housing as well as potential variations in management practices. The blood samples that are collected must be centrifuged to separate the serum that is then tested to determine the IgG concentration and if adequate passive transfer has occurred.

The final step of assessing factors affecting passive immunity is the ability to measure antibodies in the calf's serum following the ingestion of MC or CR. Radial immunodiffusion **(RID)** is currently the gold standard to determine IgG concentration in MC and bovine serum, however the incubation time for this assay can range from 18 to 48 h depending on the test kit. Cessation of antibody uptake by fetal-type enterocytes and transport of IgG into circulation ceases around 24 h of age in the neonatal calf (Devery et al., 1979; Bush 1971). This process, referred to as gut closure, prevents the uptake of intact IgG molecules and other macromolecules. Due to the long incubation time of the RID test, producers are unable to identify a failure of passive transfer **(FPT)** calf prior to gut closure. The development of a test that could rapidly and accurately determine IgG concentration of MC and calf serum would be of great benefit to producers as FPT calves could be identified early within the first day of life and be provided with additional IgG whether in the form of MC or CR.

While blood based measurements allows for an estimation of the amount of IgG absorbed into the enterocyte and then transported into circulation, it is also important to understand and be able to measure transport of IgG into the intestinal enterocyte. Intestinal transport studies in rodents and pigs have adopted the use of Ussing chambers to determine gut permeability, absorption rates, as well as factors that may potentially alter transport. Adapting and validating the Ussing chamber technique as a method to determine antibody and nutrient uptake in neonatal bovine intestinal tissue will allow researchers to rapidly and inexpensively determine specific factors that impact intestinal uptake of macromolecules without having to do large field trials. Researchers would also have the opportunity to look at individual components of MC or CR and their affects on nutrient uptake and gut permeability.

Neonatal calf management and research can be a challenge as there are many physiological changes occurring within the calf during the first day of life. Combining research that looks at methods to more rapidly allow the quantification of IgG concentration in both MC and serum will greatly benefit producers. Validating the Ussing chamber technique in neonatal calves will allow researchers to more accurately identify factors that impact IgG uptake in the GIT of the neonatal calf.

Part I: Colostrum

Colostrogenesis

Colostrum is produced during a distinct physiological and functional stage of mammary gland **(MG)** development that is markedly different from the gland's primary role of milk production (Barrington et al., 2001). In domestic ruminants, the primary difference between MC and milk is the high concentration of colostral Ig, specifically immunoglobulin G_1 (**IgG_1**). Colostrogenesis, or the prepartum transfer of IgG from maternal circulation into mammary secretions, is a discrete and finite stage of the lactation cycle (Barrington et al., 2001). Transfer of IgG begins several weeks prior to parturition and ceases abruptly immediately prior to parturition (Brandon et al., 1971). During colostrogenesis, homologous and heterologous IgG is concentrated over 100 times more than serum albumin (Goff and Horst, 1997).

The mechanism and regulation of colostrogenesis are not as well known as that of the other stages of MG development. There is clear evidence that colostrogenesis is regulated in part by lactogenic hormones, as estrogen and progesterone are necessary for the initiation of IgG_1 transfer into MC (Barrington, 2001), and is affected by local mechanisms within the MG. A simple example of this is the variation in IgG content and composition of secretions between individual quarters of the same udder (Guidry et al., 1980).

Immunoglobulin G is transferred from the bloodstream across the mammary barrier by a specific transport mechanism. The Fc receptors on the mammary alveolar epithelial cells bind IgG from the extracellular fluid and the molecule undergoes endocytosis, transport and finally release into the luminal secretions (Larson, 1980). Selective transport of IgG_1 into MC requires two separate functions (Larson, 1980). Specific Fc receptors for IgG_1 must be present on the basal plasma membrane of the secretory cells, positioned for binding of the ligand from the extracellular fluid. In addition, mammary epithelial cells must be able to internalize and transcytose IgG_1 in order to deliver it into the luminal secretions (Barrington et al., 2001). Smaller amounts of IgA and IgM are largely derived from local synthesis by plasma-cytes in the mammary gland (Godden, 2008) and a smaller amount transported from the blood (McGuirk and Collins, 2004).

Colostrogenesis ceases prior to, or at the onset, of lactation suggesting that the hormones necessary for lactogenesis are likely candidates for regulating the cessation of IgG transfer (Barrington, 2000). Winger et al. (1995) concluded that pharmacological doses of dexamethasone given to cows actively concentrating IgG_1 in mammary secretions resulted in a sharp decline in IgG_1 concentrations. This result is consistent with the accepted role of glucocorticoids as part of the lactogenic complex that triggers the onset of milk secretion. However, glucocorticoids can cause induction of premature parturition and it could be the termination of pregnancy, rather than specific hormonal effects that may account for the decrease in IgG transfer to the MG (Barrington et al., 2001). It is likely the combination of different hormone concentrations plays a significant role in regulation of colostrogenesis and lactogenesis.

Colostrum Composition:

Fetal development occurs in a protective uterine environment. Immediately after birth the neonate is exposed to a myriad of challenges, including a need for nutrients and protection from microbial pathogens. Colostrum provides the neonate with IgG essential for passive immunity as well as many other nutrients (protein, fat, vitamins, minerals and water) vital to the calf's survival (Table 1). The carbohydrates, fat, and protein in colostrum are essential as metabolic fuels to the newborn (NRC, 2001). The vitamins and minerals are essential as co-factors for enzymes and general maintenance functions. Increasing evidence in calves and other species indicate that MC also provides maternal leukocytes, growth factors, hormones, cytokines, and nonspecific antimicrobial factors, all of which are necessary to stimulate growth and development of the digestive tract and other organ systems (Davis and Drackley, 1998; Hammon and Blum, 1998; NRC, 2001).

Proteins

Bovine MC contains 14 – 26% protein (Smith, 1946; Foley and Otterby, 1978); this is much greater than the protein concentration in milk (~ 3.1% protein; Foley and Otterby, 1978). The major proteins in MC include the family of caseins, β-lactoglobulin, α-lactoglobulin, immunoglobulins, lactoferrin and various minor whey proteins such as transferrin and serum albumin. Proteins within MC not only provide nutrition for the neonate but also enhance the immune system, act as a defense against pathogenic bacteria, viruses and yeast, and are important for the development of the GIT (Bösze, 2008).

The biological properties of proteins in MC facilitate nutrient assimilation and peptides with regulatory activity likely influence the growth and differentiation of various neonatal tissues (Talukder et al., 2002). The effect of these proteins depends on the absorption and transport of colostral macromolecules from the gut of the lumen to specific tissues. Large quantities of amino acids **(AA)** are also needed for the rapid protein accretion that occurs independent of IgG accumulation in the G.I.T. (Davis amd Drackley, 1998). The majority of the MC protein composition is Ig (Akers, 2002; Foley and Otterby, 1978).

Immunoglobulins

Due to the placental structure of ruminants, calves are born agammaglobulinemic and lack antigenic sensitization, thus the young calf is unable to develop its own local immune responses that will protect its intestinal and respiratory mucosa, the first sites to be invaded and challenged by environmental antigens (Salmon, 1999). Colostral IgG has a dual protective role in the neonatal calf (Logan and Penhale, 1971). Immunoglobulin G is absorbed from the small intestine into circulation to protect against septicemia and other immune challenges, IgG that is not absorbed can remain in the intestine and have a local protective effect.

Immunoglobulins refer to a family of high molecular weight proteins that share common physico-chemical characteristics and antigenic determinants (Butler, 1969). These proteins occur in the serum and other body fluids of animals and possess γ- or slow β-electrophoretic mobility (Butler, 1969). The AA content of Ig is quite different than any other proteins found in mammalian milk or serum (Smith, 1948). All Ig are composed of polypeptide chains referred to as heavy (long) chains and light (short) chains that vary in composition of both component AA and prosthetic groups (Larson, 1980). The heavy chain of the molecule is what gives Ig their distinct biological functions (Bergmann-Leitner, 2008). The heavy chain differs between Ig isotypes and mediates binding to specific Fc receptors and/or binding and activation of complement. The classification of Ig is based on the antigenic and physico-chemical characteristics of these proteins.

There are three identified classes of Ig in cattle, IgG, IgA, and IgM. These Ig account for 85 to 90%, 5% and 7%, respectively of the total Ig in colostrum (Godden, 2008; Klaus, 1969). These different classes may share antigentic similarities, but have different physico-chemical features. In addition to class differences among immunoglobulins, smaller antigenic and physico-chemical differences in the heavy polypeptide chains within a class give rise to subclasses (Butler, 1969). In cattle, this is seen with two subclasses of IgG: IgG_1 and IgG_2. These subclasses differ slightly in their heavy chains and are in nearly equal concentrations in the blood (Larson, 1980).

Bovine IgG is the most abundant and most extensively studied immunoglobulin in the cow. Approximately 85-90% of the serum and whey immunoglobulins are in the IgG class (Klaus, 1969). The two subclasses of IgG differ antigenically and in amino acid **(AA)** composition. Immunoglobulin G_2 is found in high amounts in serum, but occurs in lower concentrations in milk, MC and saliva (Butler, 1969). Immunoglobulin G_1 is the principal Ig for passive immunization of the calf and also fixes complement (Butler, 1969).

Bovine IgM is an antigenically distinct macroglobulin, comprising less than 10% of serum and colostral immunoglobulins (Klaus, 1969). Immunoglobulin M is a more effective antibody than IgG in agglutination, phage neutralization, complement fixation and hemolysis (Butler, 1969). Much of the efficiency of IgM can be explained on the basis of size and number of antibody combining sites. Immunoglobulin M is composed of 5, 4-chain subunit disulfide bonds found on the Fc portion of their µ-heavy chain (Butler, 1969). This configuration provides IgM with 10 potential antibody combining sites.

Immunoglobulin A has been shown to be antigenically distinct from IgG and IgM by immunodiffusion. Bovine secretory IgA is composed of four alpha chains, four light chains and one molecule of glycoprotein-a (Butler, 1969). The alpha chains are antigenically and physiochemically distinct from the heavy chains of IgM and IgG, while the light chains are identical to those occurring on other bovine immunoglobulins (Butler, 1969).

Immunoglobulin A is capable of neutralizing and preventing the entry of potentially harmful antigens into the host (Bösze, 2008). Immunoglobulin A forms antigen complexes in the lumen of the intestine and prevents absorption of dietary antigens, and thus reduces their transfer into circulation (Salmon, 1999). Secretory IgA is believed to play a role in the control of allergen absorption and contribute to the protection of the host against the development of allergies of dietary or environmental origin (Welch and May, 1979).

Non-nutritional proteins and peptides:

Non-nutritional proteins and peptides, including, but not limited to, cytokines, hormones and growth factors, are present in MC at higher concentrations than found in milk, and potentially modify early G.I.T. development (Baumrucker et al., 1994; Blätter et al., 2001; Sparks et al, 2003) and immune function. Insulin-like growth factor **(IGF-I)** is present in high concentrations at the end of pregnancy, with the greatest mass available at the start of lactation (Baumrucker et al., 1993). It has been reported that IGF-I has a stimulating effect on neonatal small intestinal tissue (Baumrucker et al., 1994). When added to milk replacer and fed to neonatal calves, IGF-1 stimulates gastrointestinal mucosal growth, brush-border enzymes and intestinal DNA synthesis (Baumrucker et al., 1994). When calves were fed a colostrum extract that contained mainly non-nutritional factors (such as IGF-I), enhanced intestinal villus size and increased epithelial cell proliferation rates were observed (Roffler et al., 2003). Feeding MC at birth results in higher circulating IGF-I at 21 d of age as compared to calves that received only milk and no MC (Sparks et al., 2003).

Insulin, a hormone necessary to regulate many metabolic processes, is found in high concentrations in bovine MC (327 ng/ml), and decreases to about 48% of its initial concentration within 24 h postpartum (Aranda et al., 1991). Higher insulin concentration in MC may be involved in stimulating nutrient uptake in the neonatal small intestine.

Nucleotides and nucleosides are active in metabolism, and appear to be important in the regulation of body function (Przybylska et al., 2007). Dietary nucleotides found in MC are important for normal development, maturation and repair of the GIT in the neonate, due to the fact that rapidly growing tissue lack the capacity for de novo synthesis of nucleotides (Uauy et al., 1994).

Cytokines are an important component of MC as they are immunological hormones that help in the development of the fetal immune response (Chase et al., 2008; Yamanaka et al., 2003). At birth neonatal calf serum does not contain Interleukin 1-beta **(IL-1β)**, interleukin -6 **(IL-6)**, tumor necrosis factor-beta **(TNF-β)** and interferon gamma **(IFN-γ)**, however all are present in bovine MC and become detectable in calf serum within 12 h after being fed MC (Yamanaka et al., 2003). These cytokines are associated with a pro-inflammatory response, and may help in the recruitment of neonatal lymphocytes into the gut to aid in immune development (Chase et al., 2008). Yamanaka et al. (2003) demonstrated that IL-1β, TNF-α and IFN-γ have a stimulatory effect on the peripheral blood mononuclear cells **(PBMC)** that is greater in newborn calves then adult cattle. They concluded that PBMC from newborn calves are highly sensitive to cytokines and contribute to immunological maturation in neonates. Aside from factors that promote immune development, there are also significant quantities of cytokine inhibitors in MC. These inhibitory factors present in human colostrum, including receptor antagonists and soluble receptors might act as regulators for cytokine toxicity (Buescher and Malinowska, 1996).

Lactoferrin and other transferrin-like proteins have received attention in both bovine MC and human breast milk as potential bacteriostatic proteins. These proteins bind iron and make it unavailable for bacterial growth (Bezkorovainy, 1977). Lactoferrin in human breast milk has a powerful bacteriostatic effect on *Escherichia coli* 011/B4 (Bullen et al., 1972). Aside from its antimicrobial activity, lactoferrin plays a role in iron uptake in the intestine and the activation of phagocytes and immune responses (Pakkanen and Aalto, 1997).

At least 20 enzymes have been either purified or isolated from bovine milk (Shahani et al., 1973). These enzymes can be water soluble, associated with cream or lipids, bound to casein or be present in microsomal particles (Shahani et al., 1972). Due to essential differences between intrauterine and extrauterine environment, the neonate is exposed to oxidative stress conditions, and it is essential that MC contains antioxidants (Przybylska et al., 2007). Enzymatic antioxidants in bovine MC include lactoperoxidase, catalase, superoxide dismutase and glutathione peroxidase. Non-enzymatic antioxidants include vitamin E, vitamin A, vitamin C, lactoferrin and selenium (Przybylska et al., 2007).

Energy in Colostrum

The newborn calf is born with relatively small energy reserves, with only 3% of the body weight made of lipids. Much of this lipid content is structural and is unable to contribute to energy needs of the calf. The small amount of fat (380 – 600 g) and glycogen (180 g) in the newborn calf would be mobilized within 18 h of life in the absence of feed intake (Okamoto et al., 1986). Energy consumed and absorbed by the neonatal calf is either transformed from the chemical form and released as heat or is retained as a new tissue (Baldwin and Bywater, 1984).

Compared with human breast milk, bovine MC contains lower amounts of neutral and acidic oligosaccharides. It is believed that the primary role of oligosaccharides is to provide protection against pathogens by acting as competitive inhibitors for the binding sites on the epithelial surfaces of the intestine (Przybylska, 2007). Bovine MC also contains lower amounts of lactose (2.69%) as compared to milk (4.7%; Tsioulpas et al., 2007). Lactose in MC and milk is hydrolyzed in the small intestine by lactase into glucose and galactose; these sugars are then absorbed by a Na^+ dependent transport pathway, SGLT-1 (Hendriques and Smith, 1974).The activity of this transporter exhibits a proximal to distal gradient, activities of lactase decreases with increasing age and weaning (O'Connor and Diamond, 1999).

The fat component of MC comprises several classes of lipids, containing mono-, di -, and triacylglycerol, free fatty acids, phospholipids, glycolipids, steroids, waxes, alcanols, carotenoids and lipoproteins (Molkentin, 2000). Colostrum from Holstein cattle contains between 6.7% (Foley and Otterby, 1978) to 9.4% (Mechor et al., 1992) fat as compared to milk which contains approximately 3.6% fat (Foley and Otterby, 1978).

The gross energy content of MC can be calculated by using the kilocalorie values for lactose, non-immunoglobulin protein and fat. Davis and Drackley (1998) calculated the average energy content of MC to be 1.16 kcal/g based on previously reported caloric estimates for lactose, fat and non-Ig protein (Brisson et al., 1957). This is considerably greater than the energy content of milk which is 0.69 kcal/g. However, the energy content of MC can vary greatly depending on the fat content. The nutrients in MC that provide energy are highly digestible therefore making milk highly digestible (92 – 98%; Davis and Drakley, 1998). The energy provided by fat and lactose in MC is essential for thermogenesis and maintenance of body temperature. Aside from being an energy source, medium chain fatty acids may also play a role in providing antimicrobial protection against viral and bacterial pathogens (Isaacs, 1995; Spring et al., 2001).

Vitamins & Minerals

Fat soluble vitamins A, D, and E cross the placental barrier in minimal amounts resulting in MC being the primary source of these nutrients for the calf (Quigley and Drewry, 1998). Colostrum is the primary source of vitamins and minerals for newborn calves and many of the minerals and vitamins are more concentrated in MC as compared to milk. An example of this is α-tocopherol, which is concentrated 6 to 7 times more than in milk (1.8 to 24.7 µg/ml; Mechor et al., 1992; Rajaraman et al., 1997). This may be an evolutionary strategy to ensure supply of adequate amounts of these minerals and vitamins for the newborn calf to initiate its own metabolism successfully and for development of the digestive and immune system (Davis and Drackley, 1998).

Limited information is available regarding how fat soluble vitamins impact immune responsiveness in the newborn calf. Vitamin A is known to promote differentiation and maturation of a variety of cell types, primarily through its metabolites (Mangelsdorf, 1994). Vitamin D acts via the metabolite, 1,25-dihydroxyvitamin D to maintain calcium homeostasis in the calf (Horst et al., 1994), and vitamin E works in conjunction with selenium as an antioxidant (Herdt and Stowe, 1991).

Maternal leukocytes

Bovine MC contains between 1 X 10^6 and 3 X 10^6 cells/mL of immunologically active maternal leukocytes (Lee et al., 1980). These maternal cells are comprised of approximately 40 to 50% macrophages, 22 to 25% lymphocytes and 25 to 37% neutrophils (Liebler-Tenorio et al., 2002; Reber et al., 2005). Animals that receive MC containing maternal leukocytes develop antigen-presenting cells faster than those animals that received MC devoid of maternal leukocytes (Reber et al., 2005). It is important for calves to develop antigen-presenting immune cells quickly because they are necessary for the development of the acquired immune response to pathogens and vaccines (Chase et al., 2008).

In neonatal ovine it has been observed that maternal lymphocyte cells in MC are absorbed from the GIT and are transported via the lacteal lymph ducts to the mesenteric lymph nodes within 4 h of uptake (Sheldrake and Husband, 1985). These cells remain immunologically active and potentially transfer immunological memory and cellular activity in lambs (Tuboly et al., 1995) and calves (Donovan et al., 2007). It was also observed that maternal cells derived from blood, not MC, that were administered to lambs failed to cross the gastrointestinal border (Tuboly et al., 1995). Similar results have been reported in piglets (Tuboly and Bernáth, 2002). This suggests that other components of MC are essential for the uptake and transport of maternal cells. Maternal cells that are present in fresh MC are destroyed if the MC is frozen for future use (Donovan et al., 2007) or processed to be used in a CR or colostrum supplement **(CS)**.

Part II. Neonatal Gastrointestinal and Immune Development:

Young calves are subjected to two stressful situations that lead to physiological changes within the immune and gastrointestinal systems, birth and weaning. During both of these periods, alterations in feeding and management can impact the future productive life of the animal.

Fetal Development

Immune system

The development of the immune system progresses in small steps from conception to maturity at approximately six months of age. Fetal calves are predominately protected by the innate immune system (Figure 1). Non-immune defense mechanisms including enzymes in secretions, acids in the stomach, fatty acids in the epithelium, complement and phagocytic cells increase in their effectiveness throughout gestation. The innate immune response of the fetus is mediated by phagocytic cells and does not fully develop until late gestation (Barrington, 2001) as these cells remain at their derivation sites until being released into the blood around 130 d of gestation. By late gestation fetal neutrophils are capable of phagocytic activity. As fetal cortisol increases prior to birth, the activity of neutrophils decreases (Tizzard, 2000).

Acquired immune defense mechanisms include antibody, memory lymphocytes and effector cells. Lymphocytes develop from stem cells, are released into the blood and move to specific locations to undergo further differentiation. During the first trimester, T and B lymphocytes migrate from primary lymphoid tissue to populate the lymph nodes, spleen and mucosal lymphoid tissues (Barrington, 2001). The immunological response of the fetus to an antigen increases with the stage of fetal development. Between 45 to 175 d of gestation, fetuses infected with bovine viral diarrhea **(BVD)** become persistently infected (Grooms, 2004). By 188 to 253 d of gestation fetal lymphocytes are responsive to mitogens (Tierney and Simpson-Morgan, 1997a and b). By 120 d of gestation, fetuses can develop antibodies to parainfluenza virus 3 and to BVD by 190 d of gestation (Banks and McGuire, 1989).

Developing fetuses, and newborn calves are subject to several immunomodulatory factors. The placenta produces progesterone, prostaglandin E_2, and cytokines that affect both the dam and the near-term fetus by suppressing cell-mediated and memory T-helper 1 **(TH1)** responses and promoting T-helper 2 **(TH2)** responses and antibody production (Morein, 2002). Prior to parturition the dam is producing estrogen and cortisol, both of which have immunosuppressive effects (Jacobs et al., 2001). Within the calf, during the parturition process, the calf is producing high levels of cortisol (Mao et al., 1994). The combined effects of these hormones suppress the immune response away from a TH1 response, and promote short term TH2 responses (Figure 2; Chase et al, 2008).

Gastrointestinal Development.

The early ontogenetic development of the intestine can be divided into five phases: 1. morphogenesis, 2. cytodifferentiation and fetal development, including preparation of the epithelium for absorption of MC and milk, 3. birth and the early suckling period, 4. suckling period and 5. weaning from milk to a solid diet (Henning, 1981; Pácha, 2000). The development of the G.I.T. in utero is characterized by extensive structural and functional changes to the epithelium. Gestational age when transport across the intestine is first detected varies among species; sheep and guinea pigs have had transport detected at or before 50% of gestation, mice, rats and rabbits do not appear to develop intestinal transport mechanisms prior to the end of gestation (Budington, 1992). Human infants acquire intestinal transport as early as 9 to 11 weeks of gestation (Koldovsky, 1984).

Birth & Day 1 of life

Immune System

Although all essential immune components are present at birth, many components are in low concentration or have reduced functionality until the calf is at least two to four wks of age, and continue to develop until puberty (Reber et al., 2005). At birth primary and secondary lymphoid tissues are populated by cells that have developed independent of antigenic stimulation; the humoral components of the innate immune system are present in limited quantity, and do not function as well as in adults (Chase et al., 2008). Complement activity within the neonatal calf is approximately 50% of that in a mature cow (Firth et al., 2005). Similar to the fetus, the innate immune defenses, including barrier defenses, of the neonatal calf are crucial for survival the first weeks of life. At birth, calves are deficient in some of the steps of phagocytosis (Zwahlen et al., 1992) and poorly opsonize bacteria (Lombardi et al., 1979).

Prior to MC ingestion (1 h post partum) phagocytic activity of polymorphonuclear leukocytes **(PMNL)** is lower when compared to PMNL of 3 to 9 wk old calves (Menge et al., 1998b). In contrast, monocytes of neonatal calves exhibit an enhanced phagocytic activity, while the oxidative burst activity of PMNL and monocytes are higher in newborn calves as compared to older calves. The ability to ingest bacteria, the generation of reactive oxygen species and myeloperoxidase activity are all reduced in PMNL of newborn calves (Zwahlen et al., 1992). Colostrum consumption alters the activities of blood phagocytes, increasing the percentage of phagocytizing PMNL and monocytes, a phenomenon that is not observed in colostrum deprived calves (Menge et al., 1998b). Changes in efficiency of phagocytizing PMNL occur within 4 h of MC ingestion (Menge et al., 1998b). This increase in efficiency is potentially due to the increase in opsonization of bacteria by colostral derived IgG (Menge et al., 1998b). Oxidative burst positive monocytes are reduced within 4 h if MC is fed 1 h post partum, where as oxidative burst positive PMNL decline within the first 4 h after birth independent of MC consumption (Menge et al., 1998b).

Ingestion of MC during the first hours of life is essential for the uptake of IgG and other immune factors. An effective cellular immune response requires antigen-specific effector cells as well as antigen presenting capacity. The uptake of maternal lymphocytes from MC and successful transfer into the neonate's circulation have a great impact on the immune system of the neonate by providing early protection and helping with the development of the immune system. Maternal lymphocytes may provide immediate, transitory antigen-specific activity (Donovan et al., 2007), as well as influencing the neonate's ability to modulate responses to foreign leukocytes (Reber et al., 2005). In a study that was able to reduce the confounding factor of IgG status, calves receiving maternal cells via MC had greater development of neonatal lymphocytes, as evidenced by an increase in antigen presenting capacity (observed as up-regulation of MHC class I) as compared to calves that received cell-free MC (Reber et al., 2008). Additionally calves receiving the maternal cells via MC had lower expression of CD11a. This suggests that up-regulation of CD11a in calves fed cell-free MC was an indication of systemic inflammation or sub-clinical disease, and that calves that do not receive maternal cells via MC have an increased susceptibility to environmental stress and infectious agents. Active synthesis and secretion of antibodies in the intestinal tissue may have little value in the context of neonatal defense. However, immunocytes synthesizing IgA and IgM, but not IgG, are shown to be present at the lamina propria of the intestine as early as 4 d of age in calves (Allen and Porter, 1975).

When calves are orally dosed with bacterial antigens at 5 d of age, they respond quickly with the secretion of antibodies, thus indicating that intestinal synthesis of antibodies can interrelate with declining maternal antibodies to provide a continuum of local antibody function in the alimentary tract of the calf (Allen and Porter, 1975). Studies in older calves (2 to 6 wks) have observed that while IgG is quantitatively the primary Ig acquired by the calf from MC, very little IgG appears within the intestines to contribute to external defenses (Porter et al., 1972).

Passive Immunity:

Newborn calves are born aggamaglobulinemic, without any measurable circulating IgG or IgM. The newborn calf derives passive immunity by absorbing immunoglobulins from the colostrum (Smith et al., 1964). Passive immunity can be both local (Immunoglobulins bathing the gut lumen) and humoral (immunoglobulins absorbed from the gut into the blood; Lecee, 1984). In the calf, passively acquired immunity is of importance to the health of the calf for the long period until they are capable of making their own antibodies (Smith, 1948). Neonates can be classified into three groups based on when they acquire passive humoral immunity (Lecce, 1984). Group I mammals acquire passive immunity postpartum. These animals include pigs, horses and ruminants. Group II mammals acquire passive immunity both pre- and post- partum. These animals include mice, rats, hamsters, dogs and cats. Group III mammals acquire passive immunity prepartum. These animals include humans, primates and guinea pigs. Calves are classified as having FPT of protective colostral immunoglobulins if serum IgG concentrations are less than 10 mg/ml when sampled between 24 and 48 h of age (NAHMS, 1996, Weaver et al., 2000).

Intestinal Absorption of Macromolecules

Following birth, there are dramatic physiological changes in GIT development and maturation. The neonate must transition from dependency on maternal sources of nutrients to maintaining its own homeostasis. The kidneys assume control of electrolyte and water balance, the lungs acquire control of gas exchange and nutrient supply is converted from maternal sources to the GIT (Kurz and Willet, 1991). Transport of macromolecules across the intestinal wall within a few hours after birth represents an important transport mode that facilitates the uptake of a number of protein molecules (Pácha, 2000). The accepted model for absorption of macromolecules in rats and other group II mammals is referred to as selective absorption and has at least three phases: adherence of the macromolecule to the brush border on enterocytes, internalization of the macromolecule within the enterocytes and egress of macromolecule into the lamina propria (Lecce, 1979). Specific transport macromolecules bind to specific receptors that shuttle them across the intestinal epithelium (Pácha, 2000).

Neonates that obtain passive transfer postpartum (group I) are able to absorb large protein molecules through their intestines without prior digestion or alteration of the molecules for a short period postpartum. Nonselective transport occurs by vesicular transport of macromolecules that adhere to the surface membrane or a transported in the fluid-phase compartment of the vesicles (Pácha, 2000). Within the calf, the process of absorption is primarily nonselective, as it has been observed that a wide variety of macromolecules can be absorbed through the gut into the blood (Lecce, 1972).

Early research has suggested that Ig absorption occurred primarily in the anterior small intestine (Fey, 1972; Fetcher, 1983). Research by Logan and Pearson (1978) observed that villous epithelial cells throughout the small intestine were capable of absorbing immunoglobulins in calves that were 4 h old. The absorption of macromolecules is divided into three phases (Staley and Bush, 1985): binding of Ig by the microvillus border followed by endocytosis of the binding site and Ig, enlargement of the tubular end piece to form a vacuole, and once the vacuole comes in contact with the cell membrane, the vacuole exocytoses its contents into the lamina propria where it passes into the lymphatics or portal circulation (Fey, 1971; Staley, 1971). Colostral macromolecules including lactoferrin, transferrin, IgG and epidermal growth factor **(EGF)** are not only absorbed into the systemic circulation, but are transported into the cerebrospinal fluid in a time dependent manner (Talukder et al., 2001). Using ligated intestinal loops, Ig absorption by epithelial cells has been observed as early as 10 min after administration (Fey, 1971). To date, *in vitro* research has been unable to document transport of IgG out of intestinal epithelial cells.

Effective uptake and transport of ingested proteins is facilitated by the presence of colostral protease inhibitors that prevent proteolytic degradation from occurring (Weström et al., 1985; Weström et al.,1982). Due to the low peptic activity (Fey, 1971), pancreatic protease levels (Huber et al., 1961) and low proteolytic degradation in the first hours of life, peptide substances such as EGF and IGF can avoid digestion and reach the small intestine. The transport mechanism of milk-borne hormones and other macromolecules have not been studied as in depth as the uptake and transport as IgG (Pácha, 2000).

Gut Closure

Rapid postnatal growth of the intestine results in the replacement of fetal-type enterocytes by adult-type enterocytes (Smith, 1985) leading to gut closure or cessation of macromolecule absorption from gut to blood. It has been observed in piglets that closure occurs first in the duodenum and proceeds toward the ileum (Lecce, 1973).The consequences of maturation of the intestine include declines in the rates of uptake per villus and a redistribution of transport from along the entire crypt-villus axis at birth to just the villus tip, with minimal to no transport in the crypts (Jarvis et al., 1977). After 24 h, IgG can still be found within the enterocyte, but is not internalized (Castro-Alonso et al., 2008). Continuing to feed MC or CR after the time of gut closure can still be beneficial to the calf, as it with bathe the GIT tract and make it difficult for bacteria to attach to the intestinal wall. This "local effect" can potentially reduce the incidence of scours during the first weeks of life (Brignole and Stott, 1980; Bush and Staley, 1980).

The regulation of intestinal transport is dependent to a large extent on the junctional complex connecting enterocytes (Pácha, 2000). Tight junctions control the passive diffusion of ions and other small solutes through the paracellular pathway. Rats and other animals in group II have an extended closure time, and attempts to induce closure have been unsuccessful (Lecce, 1965/66). Neonates in group I have a short closure times, with cessation of macromolecule transport increasing after 12 h with a mean closure time of 24 h after birth (Stott et al., 1979a; Bush and Staley, 1980). When calves are fed iodine-125 labeled IgG after 36 h, none is observed in the blood, thus suggesting that closure is complete within the first 36 h of life (Devery et al., 1979). In Group I animals, closure can be influenced. It is proposed that there are two sets of signals that cause age and region specific shifts in transporter activities: external and internal signals. External mechanisms include changes in nutrient input and presence of biologically active peptides. Internal signals include genetically hard-wired timing mechanisms (Buddington, 1992; Toloza and Diamond, 1992;

Vega and Puchal, 1992) endocrine signals, paracrine signals, and neuromodulation (Carey and Cooke, 1989).

Diet, an external signal, is one of the easiest ways to manipulate intestinal morphology (Blätter et al, 2001; Seegraber and Morrill, 1985) and gut closure (Lecce, 1973). Intestinal epithelium from 3 d old piglets that were denied food from birth had nearly the same capacity to take up fluorescent Ig as at birth (Lecce, 1973). In piglets and lambs that were allowed to nurse, gut closure occurred within 36 h. In piglets and lambs not allowed to nurse the gut remained open to absorb Ig for a longer period of time (Lecce, 1965/66). Furthering this research, piglets allowed to consume greater than 300 mL of bovine MC had earlier gut closure then those consuming less MC. Further research has led to the conclusions that newborn piglets and lambs are susceptible to fasting hypoglycemia as blood glucose levels decreases after birth (Hanawalt and Sampson, 1947) and do not recover until after the first feeding (Goodwin, 1957). Lecce (1966) observed that at least 300 millequivalents of glucose were required to induce gut-closure in piglets. In newborn calves, insulin-induced hypoglycemia will delay gut closure (Tyler and Ramsey, 1993)

It is important for closure to occur shortly after ingestion of MC to prevent the uptake of bacterial pathogens that may be present in the environment. The high rates of mortality and morbidity in hypogammaglobulinemic FPT calves may potentially be due to the transepithelial migration of pathogens prior to gut closure. Gut closure in calves can be estimated by tracking the serum IgG pattern for the first 24 to 48 h of life. Following the peak in serum IgG concentration there is a gradual decline in serum concentration due to discontinued absorption (gut closure) and/or transfer of absorbed IgG to other metabolic pools (Bush, 1971).

Successful transfer of passive immunity (serum IgG > 10 mg/ml) can be determined by measuring the concentration of IgG in the serum of the calf at 24 to 48 h after birth. Calves classified at FPT are at a greater risk of disease. Measuring the percentage of calves that achieve successful passive transfer compared to calves that are classified FPT allows producers to monitor their colostrum management and neonatal calf management protocols, as well as identify calves that may need additional attention.

In 1991 to 1992, the National Dairy Heifer Evaluation Project estimated that over 40% of heifer calves in the U.S. did not achieve adequate passive transfer. Utilizing data from the 2007 USDA National Animal Health and Monitoring system it is estimated that the prevalence of FPT in U.S. dairy heifers has decreased to 19.2% (Beam et al., 2009). While this decrease suggests that improvements in management occurred, there is still a large percentage of calves that are at a greater risk for disease. Management of both newborn calves and MC needs to continue to improve.

While determining if a calf has achieved adequate passive transfer is important, it is also important to understand the apparent efficiency of absorption **(AEA)** of IgG. Apparent efficiency of absorption ties in serum IgG concentration, serum volume and amount of IgG consumed, thus allowing researchers to estimate what percentage of the IgG consumed was transported across the intestine and entered circulation.

AEA (%) = serum IgG (g/L) x serum volume (L) / IgG consumed (g)

Factors that Affect Passive Transfer and AEA

There are various factors that impact passive transfer in newborn calves during the first 24 h of life. The primary factors that influence passive transfer are the amount of quality, IgG concentration, MC fed (Stott and Fellah, 1983) and the time elapsed postpartum prior to feeding (Nocek, 1984). Other factors that influence passive transfer include environment and parturient reproductive abnormalities; this includes parity of dam, calving ease, month of birth, maximum environmental temperature on day of birth (Stott et al, 1975) sex of calf, placental retention and dystocia (Donovan, 1986).

Colostral IgG Concentration

Stott and Fellah (1983) reported that serum IgG and IgA concentrations in calves at 24 h of age had a positive linear relationship with IgG and IgA concentrations in the MC fed. Serum IgM concentrations in serum had a quadratic relationship to amount in MC. Besser et al. (1985) observed that there was a significant negative correlation between AEA and mass of Ig fed for both IgG and IgM. In this study, calves that were fed MC with less IgM, absorbed a higher proportion of the IgM compared to calves that were fed MC with a greater IgM concentration. There was also a higher AEA of IgG when less was fed; however, calves that received MC with a higher IgG concentration had higher serum IgG levels as compared to calves that received the lower amount of IgG. This information suggests that the transport system for IgM and IgG can become saturated if overloaded, and it may be more beneficial to the calf to feed smaller volumes of MC more often.

Jaster (2005) evaluated quality, quantity and timing of MC feeding on IgG_1 absorption in Jersey calves and observed that calves fed MC with higher concentrations of total ingested IgG_1 had significantly higher serum IgG_1 concentrations than calves fed MC that had low IgG_1. In addition, calves receiving 2 L of high IgG_1 MC at birth and 12 h had higher mean AEA of IgG_1 as compared to calves receiving 4 L of high IgG_1 MC at birth, calves receiving 4 L of low IgG_1 at birth and calves receiving 2 L of low IgG_1 at birth and 12 h. These data suggest that to maximize IgG absorption calves should receive two separate feedings of high quality MC. Contradictory; a separate study concluded that feeding 150 g of IgG as soon as possible after birth resulted in greater 24 h serum IgG concentrations (10.6 vs. 13.0 mg/ml, respectively) and greater AEA (30 vs. 35%, respectively) as compared to calves fed two feeding of MC 7 h apart (Hammer et al., 2004).

Time post partum

The age at first MC feeding greatly influences gut closure (Stott et al., 1979a) and rate of absorption (Stott et al., 1979b). It appears that the trend is linear, with closure time earlier for calves fed at 0 h, however, it appears that that the length of time that calves were able to absorb IgG decreased as time to first feeding increased (Stott et al., 1979a). Conclusions from the work done by Stott et al (1979 a,b and c) suggest that contact of the intestinal epithelial cells with ingested MC immediately excite pinocytotic activity, with the rapid uptake of available macromolecules and other ingested substances into the cells until the finite amount of pinocytotic activity was discharged. With the exhaustion of pinocytotic activity, macromolecular intake into the cells is discontinued. Transport and release of macromolecules continues through the remaining absorptive periods until complete or until cessation occurred due to aging and maturation of absorptive cells.

In addition to the maturation of intestinal epithelial cells during the first day of life, there is an increase in the secretion of digestive enzymes that may contribute to reducing the AEA of colostral IgG. The efficiency of IgG transfer is greatest in the first 4 h of life, and decreases after 6 h of age (Besser et al., 1985; Michanek et al., 1989). Calves that receive MC within 4 h after birth are more likely to obtain adequate passive transfer than calves fed after 4 h or calves that nurse (Beam, 2009). This same study reported that 58.7% of U.S.

heifer calves receive MC within 4 h of birth, 15.1% receive MC after 4 h, and 26.2% of heifers calves are allowed to nurse.

Seasonal Effects

Seasonal variations in passive transfer in calves have been demonstrated in several temperate regions. Mean monthly serum IgG_1 concentration were lowest in winter and increased during spring and early summer and peaked in September, when the greatest environmental stress was cold (Boyd, 1972; Gay, 1965; Gay 1983). In a similar study (Donovan et al. 1986) contradictory data was reported, however this study was done in a subtropical climate where intense heat was the greatest environmental stress. Stott et al, (1975) observed that calves exposed to hot, less desirable environments responded by having higher mortality, higher serum corticosteroid concentration and lower serum IgG_1 concentrations at 2 and 10 d after birth. When calves are born into hot environments, housing (including shade and cooling) that alters the ambient temperature can have an impact on mortality rates, serum corticosteroid and serum IgG concentrations in newborn calves (Stott et al., 1975). The variation in seasonal pattern suggests that management practices likely play an important role in the seasonal variation in IgG absorption as people may not be as motivated to spend the extra time to tend to newborn calves when the weather is at one extreme or the other (Donovan et al., 1986).

Colostrum pH

Maternal colostrum has an average pH of 6.17 (Tsioulpas et al., 2007). The chemical compositions of CR and CS may affect the AEA (Quigley et al, 2000). Quigley et al. (2000) studied the effect of varying pH on AEA of a CS derived from edible-grade bovine serum. Lifeline Calf Nutritional Colostrum Supplement (American Protein Corporation, Ames, IA) was utilized in the study and had a pH of 7.5 when reconstituted in 2 L of water. Using sodium citrate the pH of the CS was altered from 7.5 to 7.0, 6.0 and 5.0. Blood samples were obtained at 0 and 24 h and analyzed for IgG. Plasma IgG levels and AEA of calves did not differ significantly among treatments. This suggests that a change in pH of CS in the range of 5.0 to 7.5 does not alter IgG absorption. However, when sodium bicarbonate was added to colostrum derived, CR (Saskattoon Colostrum, Saskatoon, Canada) to increase the pH from 6 to 7, calves receiving the CR with sodium bicarbonate had higher 24 and 48 h serum IgG concentrations than calves that received CR without sodium bicarbonate. This suggests that the sodium bicarbonate, not the change in pH may alter IgG absorption from CR (Morrill et al., 2010). In contrast, Ayers and Besser (1992) reported that alkalizing agents (sodium bicarbonate and doxapram HCl) resulted in significant alterations of blood-gas and acid base values, but did not affect IgG absorption. Additional research in this area is currently being conducted at the University of New Hampshire and the University of Guelph to determine the optimal dose of sodium bicarbonate, if similar results are obtain using MC instead of CR and to determine if there are differences in health and growth parameters of calves supplemented with sodium bicarbonate.

Metabolic State of the Calf

Normal birth is often accompanied by a period of hypoxia or ischemia. The increase in partial pressure of CO_2 (P_{CO2}) lowers pH, resulting in mild acidosis (Garry, 1993). While metabolic acidosis usually resolves itself within 2 h of birth, respiratory acidosis may persist for greater than 24 h post-partum (Besser et al, 1990; Boyd, 1989). Birth P_{CO2} is inversely correlated to serum IgG_1 concentrations (Boyd, 1989) and postnatal respiratory acidosis, not metabolic acidosis, can adversely affect IgG absorption (Besser et al., 1990). Tyler and

Ramsey (1991) suggested that hypoxia immediately after birth may delay the absorption of IgG but not affect peak IgG concentration in the serum. Conversely, others have reported that there is not a significant relationship between P_{CO2} and plasma IgG concentration or AEA in calves (Ayers and Besser, 1992; Drewry et al., 1999: Lopez et al., 1994).

Dystocia

Dystocia, or difficulty calving, is a cause of weakness, morbidity and mortality in dairy calves. A prolonged and difficult calving can cause acidosis and hypoxia in the calf (Davis & Drakley, 1998), both of which have major detrimental effects on IgG absorption and gut closure. Donovan et al. (1986) reported that dystocia was associated with lower neonatal serum protein concentration. Odde (1988) observed similar results in a study conducted to determine the effect of body condition at calving and calving difficulty on calf vigor and calf serum IgG concentration. A calving difficulty score of one through three was assigned to each calf. Calves with a calving score of one (unassisted delivery) had a significantly higher serum IgG_1 and serum IgM concentration as compared to calves with a calving score of two or three (2401.1 mg/dl compared to 2191.0 and 1918.5 mg/dl for serum IgG and 194.8 mg/dl compared to 173.0 and 135.6 mg/dl for serum IgM, respectively).

Data from the 2007 NAHMS study further supports that dystocia increases the risk of FPT in heifer calves when veterinary assistance was not available to correctly position the calf. Supplementation of oxygen and assistance to suckle did not affect dystocia calves' ability to obtain adequate passive transfer (Beam, 2009). The reduced IgG serum concentration in dystocia calves could be related to the increase in endogenous corticosteroid release, and their effect on closure of the intestinal wall. Another explanation for this is that calves that experience dystocia often lack vigor, and may lack the ability to suckle the dam, or bottle. To help reduce FPT among dystocia calves, managers should feed any calves that do not suckle via esophageal intubation and should also seek veterinary assistance when unable to correctly deliver a calf (Beam et al., 2009).

Sex of the calf

Heifer calves have higher serum IgG_1 concentrations as compared to bull calves (2355.4 mg/dl and 2037.4 mg/dl, respectively) at 36 h of age (Odde, 1988). It was hypothesized that this was due to the higher degree of calving difficulty for bull calves (Odde, 1988). Conversely, other studies have observed no difference in serum IgG concentrations or rates of FPT between bulls and heifers (Morrill et al., 2010; Shea et al., 2009). These differences could be due to differences in management of heifer calves compared to bull calves, rather than true gender differences.

Method of feeding

The question of “what is the best method to feed calves to assure adequate passive transfer” has been a common question in the dairy industry for over 30 years. Early research had suggested that allowing the calf to suckle from the dam may increase efficiency of colostral IgG absorption (Stott et al, 1979d). However, challenges of weakness of the cow from parturition, udder size and teat shape as well as weakness of the calf may prevent adequate suckling (Broom, 1983). In addition, the volume of MC consumed by suckling calves is often inadequate to achieve passive transfer of IgG (Stott et al., 1979d). Due to the increase in risk of disease transmission through MC as well as lack of certainty in regard to the volume of MC the calf is ingesting has led to the recommendations to not allow calves to nurse from their dam. The use of artificial feeding systems (bottles, nipple buckets or esophageal feeders) has allowed producers to dictate the volume of MC or CR being fed to the newborn calf.

Besser et al. (1991) conducted a study to determine the rate of FPT based on feeding method. Calves having to suckle (n = 165) had an FPT rate of 61.4%. Failure of passive transfer rates were greatly reduced when calves were fed via esophageal tube (n = 33) or bottle (n = 83) to 10.8% and 19.3%, respectively. Mean IgG concentration across the three treatment groups was not different; however it is difficult to determine the exact concentration and quantity of IgG consumed by suckling calves.

Competitions between macromolecules

One factor that is often overlooked in MC management is that there is competition between macromolecules and microorganisms for uptake into the intestinal enterocyte (Staley and Bush, 1985). *Escherichia coli* **(*E.coli*)** attach to the intestinal epithelium of calves (Corley et al., 1977). The binding of *E. coli* to the intestinal epithelium is believed to have a suppressive effect on endocytosis and cellular vacuolization of immunoglobulins (James et al., 1980). Bacteria in MC can also bind free Ig and block its uptake across the intestine (James et al., 1978, 1980)

Failure to achieve adequate passive transfer increases the risk of neonatal septicemia and mortality (Nocek et al., 1984) as well as decreasing long-term profitability. Bush and Staley (1980) hypothesized that a certain frequency of hypogammaglobulinanemia cannot be avoided under practical farm conditions due to variations in birth weight, IgG concentration in colostrum, dose of MC, age at first feeding and possibly to genetically determined ability to absorb IgG. If FPT occurs, calves can be treated with the administration of plasma at a dosage of 20 mL/kg I.V. (Weaver, 2000). This practice is often not an option, however;

calves that suffer FPT can still survive if they are placed in a clean environment with low exposure to infectious pathogens (Weaver, 2000). It is also important to remember that neonates that do not achieve adequate passive transfer can easily succumb to a disease state if placed in a dirty environment or are exposed to bacterial pathogens or other disease-causing organisms.

Colostrum Quality

The concentration of IgG in MC is important in determining the amount of IgG the calf consumes and can potentially absorb into circulation. Colostral IgG concentration is the primary factor that can affect passive transfer (Nocek et al., 1984), and is the hallmark for evaluation of MC quality. Immunoglobulin concentration in MC is not uniform across cattle and can range from 1.7 to 8.7% on a whole MC basis (Bush, 1971). High quality MC has an IgG concentration greater than 50 g/L (Godden, 2008). Various factors can affect the concentration of IgG and nutrient composition of MC; these factors include, but are not limited to, parity, quantity of MC produced, breed, environmental and management strategies.

Parity

Second and later lactation cows have higher IgG concentrations in MC as compared to first parity cows (Devery-Pocius and Larson, 1983; Shearer et al., 1985). Devery-Pocius and Larson (1983) observed that total IgG_1 reached a maximum concentration in the third and fourth lactation and nearly doubled as compared to the first lactation. Total IgG_2 was lowest in first lactation cows and increased with lactation number, however, only MC from cows in lactation number five to eight had significantly greater values compared to MC from lactation one animals. Serum IgM and IgA did not show any trend with age. Quigley et al.

(1994) observed that IgM increased linearly as lactation number increased, and IgA was higher in MC from second lactation cows than from first lactation cows or cows in third or greater lactations.

Immunoglobulin G is transported from the blood to the MG by a highly specialized transport system. The increase in IgG concentration of MC as parity increases suggests that the transport system may not mature until later lactations, and is coincident with maximum MG development. Lower IgG concentrations in MC from first lactation animals could occur because first parity cows have been exposed to fewer antigens than older cows and therefore produce lower quantities of antibodies that can be transported to the MG.

Quantity

A second factor that can impact the IgG concentration of MC is the quantity of MC produced at first milking. Weight of first milking postpartum is the variable most highly correlated (negatively) with colostral IgG_1 concentration (Pritchett et al, 1991). The IgG becomes more diluted as the quantity of MC produced increases, thus, more MC must be fed for the calf to ontain adequate colostral IgG and achieve successful passive transfer.

Breed

Guy et al. (1994) investigated the physiological basis of breed differences in IgG_1 concentration in MC. Utilizing 15 beef cows and 13 dairy cows; overall IgG_1 concentration was greater in colostral secretions from beef cows (113.4 mg/ml) than from dairy cows (42.7 mg/ml). The conclusion from this study was that dairy cattle transfer more IgG_1into secretion than beef cattle, but colostral IgG concentrations were lower in dairy cattle due to the dilution effect of the greater volume of MC produced. Muller and Ellinger (1981) compared total IgG content of MC among various breeds of dairy cattle, Holstein cows produced MC with total

Ig content (5.6%) that was numerically lower than for Guernsey (6.3%) and Brown Swiss cows (6.6%), and was statistically lower than Ayrshire (8.1%) and Jersey cows (9.0%). These differences could be attributed to genetic differences and/or dilution effects.

Climate/Environment

One factor that potentially effects MC composition is temperature and humidity. While adequate ventilation and cooling systems help reduce the effects of climate, it still remains a viable factor affecting MC quality. Nardone et al. (1997) reported that when primiparous cows were exposed to high air temperatures (temperature – humidity index = 82 from 0900 to 2000 h and temperature humidity index = 76 from 2100 to 0800 h) it markedly affected MC composition, as compared to cows exposed to thermal comfort (temperature – humidity index = 65). For the first four milkings, MC from cows under high air temperatures had lower mean percentages of total protein (7.5%) as compared to their herdmates who were exposed to thermal comfort (9.0%). Further analysis of colostral protein fractions showed that heat stress reduced the concentration of casein, lactalbumin, and IgG, and IgA, but did not reduce the percentage of lactoglobulin or the concentration of IgM.

Management Factors

Management factors can also play a role in altering MC composition and IgG concentration. New mastitis infections often occur during the dry period, and this can negatively impact MC quality. Gulliksen et al. (2008) reported that a somatic cell count **(SCC)** greater than 50,000 cells/mL was the only test-day result found to be significant for the production of MC with very low IgG values. Ferdowaki et al. (2009) reported that when calves were fed Mc with a high SCC, calves had reduced serum IgG concentrations, greater incidences of diarrhea, and compromised health status during the first 42 days of life.

Mastitis infections can be prevented during the dry period by proper management of dry cow and close up pens and with the use of antibiotics at dry off and teat sealants to prevent bacteria and other microorganisms from entering the udder. It is currently recommended that MC collected from cows with mastitis should be discarded (McGuir and Collins, 2004).

Milking pre-partum heifers and cows has become a common management practice on some dairies as a way to reduce udder edema and prevent mastitis in older cows. While this practice can be beneficial to the cow, it reduces the quality of MC. Transfer of IgG into milk is largely completed prior to parturition. Pre-milking cows, or cows that have excessive leakage prior to calving, will result in a loss of IgG from the MG, and will result in MC with lower IgG concentrations (Kruse, 1970). If pre-partum milking is a management practice, producers should maintain a supply of quality frozen MC or CR that can be used if adequate, fresh, MC is not available.

Colostrum management does not stop once MC is harvested from the cow. Colostrum provides a great media for bacterial growth and during storage at ambient temperatures colostral pH decreases, TS, protein, fat and lactose decrease and microbial numbers rapidly increase (Foley and Otterby, 1978). Current recommendations suggest not to feed MC that contains greater than 100,000 cfu/ml total bacteria count **(TPC)** or greater than 10,000 cfu/ml total coliform count (McGuirk and Collins, 2004). To prevent/reduce bacterial contamination of MC, managers should properly prepare udders prior to harvesting MC, milk into a clean, sanitized bucket, and then handle MC with clean, sterilized storage or feeding equipment (Godden, 2008).

Determining Colostrum Quality

It is commonly recommended that the minimum mass of IgG fed in the first feeding should be no less than 100 g for a calf to obtain adequate passive transfer (Davis and Drakley, 1998). In order to feed an adequate amount of IgG, one must know the IgG content of the MC, and adjust the volume fed to reflect the amount (g) of IgG necessary for the calf to obtain adequate passive transfer. Colostrum quality is most commonly estimated based on appearance and apparent viscosity even though these methods are very subjective and often inaccurate. It is highly recommended that producers do not feed calves MC that is visibly watery, bloody or from cows that have leaked before calving (Godden, 2008). It is also not recommended to feed MC to calves from cows and heifers that have tested positive for bovine leukosis, *Mycobacterium paratuberculosis, E. coli, Mycoplasma bovis* mastitis, *Staphylococcus aureus* mastitis, BVD, or other diseases that can be shed and passed via MC (McGuirk and Collins, 2004).

The colostrometer was introduced as a practical field tool for measuring IgG concentration in bovine MC based on the linear relationship between colostral specific gravity and IgG concentration (Fleenor and Stott, 1980). The colostrometer is calibrated to IgG concentration at intervals of 5 mg/ml from 0 to 180 mg/ml, displayed with 3 color coded quality regions: poor (red) less than 22 mg/ml, moderate (yellow) 22 to 50 mg/ml and excellent (green) greater than 50 mg/ml. The colostrometer is not an analytical technique, but rather a method that can be utilized to estimate relative quality of MC. While colostrometers have allowed producers to identify poor or quality MC, they are often inaccurate (Morin et al., 2001). Colostrometers often overestimate IgG concentration (Mechor et al., 1992) or are utilized improperly (Mechor et al., 1991, 1992). The quality readings on the colostrometer are based on the specific gravity of normal milk and provide an estimate of relative quality, not actual IgG quantity (Fleenor and Stott, 1980; Quigley et al., 1994). Colostral specific gravity can be affected by breed, month of calving and parity (Morin et al., 2001).

Refractometers are another readily available tool that can be utilized to measure the total protein **(TP)** content in MC (Moore et al., 2009). Protein solutions refract light, and refractometers use this property to measure TP in a solution (Chavatte et al., 1998). A refractometer can provide a reasonable estimation (R^2 = 0.76) of IgG concentration when MC is measured at 20^0C (Mechor et al., 1992). Refractometers have been used to estimate the IgG content in colostral whey (Molla 1980), and TP in whole MC. Bielmann (2010) reported that optical and digital brix refractometers were highly correlated for both fresh and frozen MC samples and had a correlation coefficient between 0.71 and 0.74 when compared to IgG concentration determined by RID. Sugar and alcohol refractometers have been used to estimate colostrum quality in mares (Cash, 1999; Chavatte et al., 1998). A brix reading of less than 10 to 15% was correlated to poor equine colostrum with a measured IgG concentration of 0 to 28 g/L, where as a brix reading greater than 30% was highly correlated to equine colostrum with a measured IgG concentration of greater than 80 g/L. Measurements obtained with the sugar and alcohol refractometers were highly correlated with the RID obtained IgG concentration (R = 0.98 and R = 0.99, respectively) in mares (Cash, 1999).

Chigerwe et al. (2008) compared four methods (weight at first milking, two hydrometers and a digital refractometer) for MC analysis to RID- obtained IgG concentration. The cutpoint for the two hydrometers were 70 and 87.5 g/L. Above these points the sensitivity and specificity of the hydrometer to accurately classify quality colostrum (MC with IgG > 50 mg/ml) decreased. For the refractometer, a brix reading of 22% was the cutpoint to accurately estimate quality MC and 8.5 kg was the cutpoint for weight at first milking. Weight at first milking was inversely related to IgG concentration of MC, however it should not be used to determine MC quality as it was poorly associated with IgG content ($R^2 = 0.03$). Both hydrometers and the refractometer had linear relationships with IgG concentration ($R^2 = 0.41$, 0.30 and 0.41, respectively). Using a refractometer allows for an accurate and rapid estimation of the TP content of MC. Developing a method that allows for the removal of the non-antibody proteins from MC should significantly improve the value of the refractometer as a method to determine MC quality.

In addition to the colostrometer and refractometer, there are laboratory techniques that allow for accurate quantification of IgG concentration in MC. Unfortunately laboratory analyses of MC are expensive, often not available to producers, and can take up to 48 h to complete, well beyond the time of gut closure. Radial immunodiffusion has been used for quantification of IgG in blood serum and plasma as well as in MC. Different methodologies used to prepare the MC for analysis can impact the final result (Fleenor and Stott, 1981). When whey was analyzed by RID the results were consistently high, and results for whole or fat free MC were low. This research recommended that whole MC be used for RID analysis.

Currently only 13% of US dairy operations evaluate MC quality prior to feeding (NAHMS, 2007). Farms that have a herd size of 500 head or more are more likely to evaluate MC quality (45.2%) as compared to farms with less than 100 head (7.6%). The most common methods to determine MC quality are the use of a colostrometer (43.7% of operations) and visual appearance (41.6% of operations; NAHMS, 2007).

How to Improve Colostrum Quality

Colostrum is vital to the survival of the newborn calf, however high quality MC is not always available. To be classified as MC of satisfactory quality, international recommendations set a minimum concentration of 50 g of IgG/L (Besser et al., 1985; Besser et al., 1990). In a study done utilizing Norwegian Red cattle, 1,250 MC samples had IgG concentrations ranging from 4 to 235 g/L with 57.8% of the samples having less than adequate IgG concentration (Gulliksen et al., 2008). Whether the cow is down, has tested positive for a disease that is transmittable via MC or it is the middle of the night, the newborn calf needs to receive colostrum within the first few hours after birth. Research has been done to improve MC, to create CS that can be added to MC and to develop CR, which can be used as a total replacement for MC.

Pasteurization

The primary purpose for pasteurization is to reduce the bacterial load of whole MC. Pasteurizing MC at 60^{o} C for 60 min is sufficient to minimize IgG loss while eliminating or reducing *E. coli, Salmonella enteritidis, Mycoplama bovis and Mycobaterium avium* (Godden, 2008) and decreasing the degree of severity of *Mycobacterium avium spp. paratuberculosis* infection in young calves (Stabel, 2008). It had previously been reported that pasteurization of MC reduced the IgG concentration (23.6 to 58.5%), and that this loss of IgG could be reduced by pasteurizing small batches of MC (Godden et al., 2003). When calves were fed 2 L of fresh MC (n = 40), or 2 L of pasteurized MC (n = 55) at first feeding, calves receiving fresh MC had higher serum IgG concentrations (19.1 mg.ml vs. 13.5 mg.ml). There was no difference in 24 h serum IgG concentration (16.1 mg/ml and 13.5 mg/ml) when calves received 4 L of fresh MC (n = 8) or 4 L of pasteurized MC (n = 20)

(Godden et al., 2003). This research suggests that on-farm pasteurization of MC may be a useful method to reduce bacterial loads of MC. However, a greater volume of the pasteurized MC should be fed to account for potential IgG loss. Once pasteurized, MC should be fed immediately as bacterial growth can resume. The long term benefits of feeding pasteurized MC to neonatal calves for the purpose of disease reduction have not been fully addressed. It is estimated that less than 1% of U.S. calves receive pasteurized MC (Beam, 2009).

Bovine Serum Products

Bovine serum products are one of the least expensive sources for CS, because bovine blood is plentiful and considered a waste product from slaughter houses and also contains high levels of IgG. Arthingon et al. (2000a) compared the AEA of IgG from a commercial bovine serum product, bovine MC (positive control), and two commercial milk derived CS. Plasma IgG concentrations at 24 hours were 12.1, 6.8, 2.2, and 3.5 g of IgG/L for bovine MC, bovine serum, CS-1 and CS-2. While calves that received bovine MC had a higher plasma IgG concentration, AEA was greatest for the calves receiving the bovine serum product. This is a direct relation to the initial amount of IgG fed to the calves. The results of this study indicate that when MC is not available, serum derived IgG may be used to supplement calves with a concentrated source of IgG.

A separate study investigated the effect of serum-derived Ig source. Calves received pooled MC, spray-dried bovine serum or spray-dried porcine serum (Arthington et al., 2000b). Calves that received the bovine serum had higher 24 h serum IgG concentrations than those receiving the pooled MC or porcine serum. This study also investigated the effect of supplementing bovine MC with varying quality with bovine serum on Ig absorption. Serum IgG concentrations at 24 h of age were greater for calves that received medium or low quality MC supplemented with bovine serum, as compared to calves that received high quality MC. Results from these studies indicate that spray-dried bovine serum is efficiently absorbed by newborn calves, and supplemental bovine serum added to medium or low quality MC is an effective method to improve passive transfer rates in calves.

Colostrum Supplement

Colostrum supplements were developed due to the high number of calves that had FPT and to address the lack of high quality MC available to feed calves. Colostrum supplements are designed to provide additional IgG (typically 25 to 45 g/dose) to neonatal animals during the period of macromolecule transport (Davenport et al., 2000). Current CS provide exogenous IgG from bovine lacteal secretions, eggs or bovine serum and are intended to provide less than 100 g of IgG/dose. Colostrum supplements are not formulated to completely replace MC (Quigley et al., 2001); therefore they may not contain other essential nutrients.

Hopkins and Quigley (1997) performed a study to determine if the addition of a CS to MC affected serum IgG concentration and AEA. Fifty- two Holstein heifer and bull calves were blocked by sex and randomly assigned to receive 3.8 L of MC in one feeding, 1.9 L of MC in two feedings or 1.9 L of MC in two feedings plus 272 g of CS at the first feeding. Blood was collected at 0, 24 and 48 h and analyzed for IgG. Serum IgG concentrations were lower ($P < 0.01$) for calves that received 1.9 L of MC in two feedings plus 272 g of CS at the first feeding as compared to calves that received 1.9 L of mC in two feedings. However, serum IgG concentrations at 48 h did not differ among treatments. The researchers suggested that if high quality MC is available to be fed, addition of CS appears unnecessary. Additional research has demonstrated that calves fed CS (90 g of IgG in 2 feedings) tend to have lower body weight gain from 0 to 60 d compared to calves receiving MC, and have significantly lower 24 h serum IgG concentrations compared to calves receiving CR. (Quigley et al., 2001).

Colostrum Replacer

While MC provides the calf with antibodies necessary to obtain passive transfer, it can also be the first exposure of pathogens to the calf. Pathogens such as *E. coli, Mycobacterium avium, Mycoplasma bovis* mastitis and bovine leucosis can all be transmitted from cow to calf via MC (McGuirk and Collins, 2004). Early exposure to these pathogens represents significant health risk and economic loss. Colostrum replacers were originally created as a way to provide calves with the needed antibodies when high quality MC was not available, or when it is known that a cow carries a specific disease that can potentially be transmitted via MC. In a recent study, 94.7% of dairies fed MC to their calves; the remaining 5.3% fed a CR most often due to the risk of Johne's disease (Fulwider et al., 2008).

Colostrum replacers must provide an adequate mass of IgG (greater than 100g of IgG/dose), for the calf to achieve a predicted final serum IgG concentration of greater than10.0 mg/mL (Quigley et al., 2001, 2002). A CR must also provide the nutrients required by the calf to thermoregulate and establish homeostasis; this includes adequate protein, energy, vitamins and minerals. The IgG in CR can be derived from bovine blood, whey, milk or MC. While CR are able to provide IgG and reduce the risk of disease transfer, they do not contain maternal-derived cells, or antibodies to farm- specific diseases.

Swan et al. (2007) studied passive transfer of IgG and pre-weaning health in Holstein calves fed MC (76.7 $\pm$ 30 mg/ml IgG) or CR (125 g IgG; Acquire, APC Inc. Ankeny, IA). Serum IgG and TP concentrations were greater (P < 0.001) for calves that received MC (14.8

± 7.0 mg/mL and 5.5 ± 0.7 g/dL) as compared to calves that received CR (5.8 ± 3.2 mg/mL and 4.6 ± 0.5). Rates of FPT were greater ($P = 0.002$) in calves that received the CR as compared to MC fed calves (93.1 and 28%, respectively). Additional studies have been done to compare MC to CR and similar results were observed (Smith and Foster, 2007). In contrast, there have also been studies done were CR were able to provide adequate passive transfer (Campbell et al., 2007: Shea et al., 2009; Morrill et al., 2010)

Shea et al. (2009) performed a study that compared the passive transfer of calves being fed different amounts of CR (Saskatoon Colostrum Company; Saskatoon, Canada) with or without supplemental lactoferrin (Immuncell; Portland, ME). Calves that were fed 1 dose of CR (105 g IgG) without supplemental lactoferrin within 90 min of birth, had lower 24 h serum IgG concentrations than calves that received 2 doses (1 dose within 90 min of birth and a second dose at 12 h) of CR (210 g IgG) without supplemental lactoferrin (10.7 and 14.4 g/L, respectively).

When calves were fed one dose of CR (132 g of IgG; Alta Gold Colostrum Replacer, Sasksatoon Colostrum Company) at birth and one-half dose (66 g IgG) at 6 h, mean 24 h serum IgG concentration on 20 calves was 13.2 g/L (Morrill et al., 2010). When sodium bicarbonate was added to the CR, 24 h serum IgG concentrations were increased to 16.3 g/L. The AEA of the CR was increased when sodium bicarbonate was added to the CR (31.2 vs 26.1%). This research suggests that feeding 1½ doses of CR (198 g of IgG) over the first 6 h provides enough IgG for the calf to achieve adequate passive transfer, and that the addition of sodium bicarbonate to the CR improves the absorption of IgG. Godden et al., (2009) reported that feeding 200 g of IgG to calves within the first 2 h of life provided a 24 h mean serum IgG concentration of 19.0 mg/ml.

These studies suggest that CR can be utilized as a management tool to replace MC and calves can achieve adequate passive transfer when provided CR within the first hours after birth. While CR and CS can be successfully used as a management tool, they do have some limitations. The profile of antibodies in the CR or CS may vary from the antigenic reservoir on individual dairies. Although the calf is absorbing IgG and achieving adequate passive transfer, there may be little protection if the IgG being absorbed is specific to pathogens not present on the dairy. A second limitation of CR and CS is that through the processing steps, pasteurization and spray-drying, activity of important hormones, colostral lymphocytes and other colostral components may be negatively impacted.

Part III – Techniques to Measure Intestinal Transport:

Introduction:

To accurately determine how and how much of a nutrient is transported across the intestine, there must be a method or model to measure the process. There currently are both direct and indirect methods to estimate absorption of nutrients and antibodies into the bloodstream and methods to estimate transport across the intestinal epithelium. Blood samples can be utilized to estimate the percentage of a nutrient absorbed based on the increase in blood or serum concentration over a defined time period. Labeled metabolites can also be used to determine rates of uptake and incorporation into tissues. While these methods are valuable, they do not allow researchers to quantify what is absorbed into the enterocyte, but not transported into circulation. Accurately determining intestinal transport and what alters transport can be challenging. Studies to determine intestinal transport of nutrients have evolved from *in vivo* procedures utilizing anesthetized animals to *in vitro* procedures utilizing intestinal loops to *ex vivo* procedures that allow for a more controlled environment to determine transport capabilities.

As advancements in knowledge and technology are made, new techniques and models must be validated across species and age groups. Modifications continue to be developed that make these techniques simpler and less invasive, while simultaneously increasing our knowledge of intestinal transport mechanisms. These advances will allow researchers to perform more extensive studies that use fewer animal subjects and can be completed over a shorter course of time. The following section will discuss both blood and intestinal-based methods to estimate absorption of IgG.

Blood Based Tests for IgG Quantification:

Quantifying IgG, IgM and IgA concentration in serum is used to investigate and determine immune system status and exposure to infectious diseases in both humans and animals. Determining the IgG status of the newborn calf is valuable to determine if adequate passive transfer has been achieved. These tests can also be used to determine the AEA of a new CR or CS, as well as MC. There are numerous lab-based tests that can directly or indirectly determine IgG concentration in plasma, serum and/or colostrum. Currently there are no calf-side tests to rapidly and accurately assess IgG status quantitatively prior to gut closure.

Blood based methods can be broken into two groups: direct and indirect methods of measurements. Direct methods are able to measure the actual concentration of a specific metabolite or antibody in the serum or plasma sample. These analyses are able to provide quantitative results to researchers and producers. Indirect methods do not measure the actual quantity or concentration of a specific metabolite or antibody, but rather provide an indication of how much may have been absorbed.

Radial Immunodiffusion Assay

Radial immunodiffusion is a direct measurement of IgG concentration and is considered to be the gold standard to determine IgG concentration in bovine serum. Radial immunodiffusion is an antibody-precipitant technique for the quantification of specific proteins in a complex mixture without separating the individual proteins to be measured (Guidry and Pearson, 1979). Specific antibody to the protein to be measured is mixed into a gel and layered on a slide or plate and wells are punched into the gel. Serum or diluted MC, as well as the internal IgG standards of the kit are placed in individual wells on the RID plate. Plates are incubated for a defined time depending on the manufacturer's guidelines. The mixture of proteins in the solution of interest (serum or MC) diffuses radially into the gel until the protein of interest reaches equilibrium with its specific antibody in the gel. At equilibrium an antigen-antibody precipitate ring is formed (Guidry and Pearson, 1979). After the recommended incubation period the precipitin ring diameter is measured. The IgG concentrations of samples are determined by comparing the diameter of the precipitation ring with a standard curve generated by the internal standards of each kit.

Strengths and Weaknesses:

Radial immunodiffusion kits are commercially available, require a very small sample of MC or serum (3 – 5 µl), and require minimal equipment to perform (Guidry and Pearson, 1979). It is suggested that whole MC be used to determine the IgG concentration by RID analysis, as removal of casein and fat from MC, or using whey from MC, increases the concentration of Ig, thus resulting in an over-prediction of total Ig (Fleenor and Stott, 1981).

A weakness in RID analysis is that discrepancies in IgG concentration can occur between RID kits made by different companies (Ameri and Wilkerson, 2008; Lee et al., 2008). These differences are potentially due to inaccuracies of the internal IgG standards that are provided with the kits. Different kits can have different thresholds in the minimum and maximum concentration of IgG that it can measure. Kits are species-specific; bovine IgG assays are not sensitive to camelid or goat IgG and vice versa. Additionally, if samples fall outside of the range of the internal standards, the standard curve should not be extrapolated. These samples should be re-analyzed in a more dilute or concentrated form to reduce the introduction of error. The greatest challenge preventing RID assays from being readily used on farm for the detection of FPT and identification of quality MC is the incubation time. The RID assays require a relatively long incubation time (~24 h) that prevents the identification of FPT calves prior to gut closure.

*Enzyme linked immunosorbent assay **(ELISA)***

Similar to RID, ELISA assays directly measure serum Ig concentration. The use of ELISA for the direct measurement of serum IgG to diagnose FPT resulted in 94% agreement with RID in a large calf study (n = 115; Lee et al., 2008). The dispersion of differences between ELISA and RID was greater at higher concentrations of IgG (> 10 mg/ml). This suggests that the specificity of the ELISA is reduced at the cutoff point of 10 mg/ml, and that the ELISA may be a useful tool to identify FPT calves, but not to quantify IgG concentration in MC.

The ELISA procedure is fairly simple. Microwells of a polystyrene plate are pre-coated with antigen, incubated for 1 h and then washed. The test sample and standards are then added to the wells and incubated for 1 h. During this incubation the antibody in the sample will bind to the antigen layer. After 1 h the plate is washed to remove any un-bound sample and a detection antibody is added to each well and again incubated for 1 h. During this time the detection antibody binds to the antigen-antibody complex in the well. After 1 h the plate is washed again and the enzyme substrate to the detection antibody is added, this leads to a color change that is proportional to the amount of bound antibody. The color intensity can be determined by spectrophotometry. Similar to RID analysis, a standard curve is created using commercially available Ig standards.

The quantitative ELISA protocol consists of many steps, however the analysis time (4 h) is still much less than that of RID analysis of serum for IgG concentration (Lee et al., 2008). The large number of tests per ELISA plate and low cost per test make ELISA suitable for analyzing a large number of samples in a research setting. This analysis is not suitable for on-farm analysis due to the number of steps and specialized laboratory equipment necessary to perform the analysis.

Turbidimetric immunoassay (TIA)

The sodium sulfite turbidity test is a 3-step semi-quantitative test, using 14, 16 and 18% solutions of sodium sulfite. Each solution causes select precipitation of high molecular weight proteins, specifically Ig, resulting in turbidity, the measured end point (Pfeiffer and McGuire, 1977). An aliquot of serum (0.1 ml) is added to a 13 x 100 m borosilicate test tube containing 1.9 ml of the sodium sulfite solution and mixed. The tube is then allowed to incubate for 15 min at 23^{o}C, and then examined for turbidity. A lack of turbidity is indicative of FPT, and appearance of turbidity is indicative of adequate passive transfer.

The sodium sulfite turbidity test has a specificity of 53% when compared to RID determination of passive transfer in calves (Dawes et al., 2002). This method did not incorrectly classify any calves as adequate passive transfer, however it did incorrectly classify 49 calves (n = 119) as FPT. In a study using 210, two year old goats, it was determined that temperature affects test results (Aba-Adulugba et al., 1989). When samples were incubated at 4^{o}C or 38^{o}C, clear and rapid precipitation occurred in 96 and 98% of samples; however, when samples were incubated (1 h) at tropical room temperature (28 to 30^{o} C), using the same salt concentration (14%) only 52% of the samples provided clear and rapid precipitation. This suggests that a consistent incubation temperature is required for consistent results.

The zinc sulfate precipitation test, like the sodium sulfite test, is a semi-quantitative assay of the globulin fraction in serum (Rumbaugh et al., 1978). Serum is added to a 0.025% zinc sulfate solution, mixed and allowed to incubate. Serum Ig will precipitate at 400-500 mg/dl (Rumbugh et al., 1978). The turbidity of the solution can then be subjectively rated from 0 (no precipitate) to 3 (heavy precipitate). The zinc sulfate turbidity test has a sensitivity of 100%, 40% specificity and 73% accuracy in 204 calves (McVicker et al., 2002). In foals, a modified zinc sulfate turbidity test in which the incubation time was shortened from 1 h to 15 and 60 seconds correctly identified 84% of FPT foals, and 100% of adequate passive transfer animals (LeBlanc et al., 1990). Reducing the incubation time also reduced the number of foals that were inaccurately classified as having adequate passive transfer, indicating that as incubation time increases, proteins other than IgG may be precipitated.

The zinc sulfate and sodium sulfite tests are indirect, semi-quantitative tests; however, they are quick, inexpensive and practical methods to evaluate if adequate passive transfer has occurred in both calves and foals. Both of these tests rely on the measurement of turbidity, which can be done by photometry or assessed visually, thus allowing the procedure to be adapted to on-farm use. When assessed visually, the reading becomes subjective and can therefore vary between technicians.

Lateral-flow immunoassay

The lateral-flow immunoassay provides a visual result of one (IgG concentration greater than 10 mg/ml) or two (IgG concentration less than 10 mg/ml) red lines as a method to determine passive transfer. Serum is mixed with a buffer, and then several drops are applied to the lateral-flow assay and allowed to incubate. When the IgG concentration is greater than 10 mg/ml, it forms a complexing agent on the strip and migrates through the test strip, bypassing an immobilized detection line, but reacting with an immobilized control line to cause a single red line to develop. When the IgG concentration is less than 10 mg/ml, it does not completely form the complex with the complexing agent. The unattached complexing reagent then migrates through the strip and reacts with the immobilized detection and control lines, resulting in 2 red lines (McVicker et al., 2002). In a study utilizing 204 calves, the lateral flow immunoassay had 95% accuracy when compared to RID-determined passive transfer results, and 99% sensitivity and 89% specificity (McVicker et al., 2002).

Immunocrit

Another indirect method to determine IgG status within animals is the immunocrit assay. This assay uses ammonium sulfate to precipitate IgG in a capillary tube. The immunocrit ratio is then determined by measuring the precipitate (mm) and the solution (mm) in the tube after centrifuging for 5 min (Vallet et al., 2010). In calves ($n = 96$) there was a strong correlation between the immunocrit ratio and RID determined IgG concentration ($r = 0.90$) when 24 h serum samples were analyzed (Vallet et al., 2010).

Gamma Glutamyltransferase

Gamma glutamyltransferase (GGT) is a membrane associated protein involved in AA transport. In MC, GGT catalytic activity concentration is 2.5 to 3.3 higher than in milk and about 300 times higher than in serum (Sobiech et al., 1974). Variations of serum GGT in the first days of life can be indicative of MC intake (Braun et al., 1982; Howard et al., 2005). Early variations of plasma GGT activity of calves has been correlated with increase of serum proteins from MC intake. Furthermore, when calves received boiled milk or milk replacer, GGT activity did not increase (Braun et al., 1982).

Gamma glutamyltransferase absorption in the neonatal intestine is limited to the same 24 h window as colostral IgG, consequently, serum GGT levels may be used to indicate adequate passive transfer when IgG concentrations cannot be measured directly (Zanker et al., 2001). Serum GGT levels increase following MC intake and then decrease substantially over the first week of life, therefore models have been used to estimate serum GGT activity that is equivalent to a serum IgG concentration of 1,000 mg/dl at different ages. At one day old, calves should have serum GGT activities greater than 200 IU/L, four day old calves should have serum GGT activities greater than 100 IU/L, one week old calves should have serum GGT activities greater than 75 IU/L. Calves below these levels should be classified as FPT (Parish et al., 1997). The greatest benefit of the method is that measurement of GGT levels in serum is not affected by hydration status (Parish et al., 1997).

Serum total protein

Refractometers, digital or optical, can be utilized to measure the TP content in MC and calf serum (Calloway, 2002; Moore et al., 2009; Bielmann, 2010). Protein solutions refract light, and refractometers use this property to measure TP in a solution (Chavatte, et al., 1998). In the neonatal calf, Ig constitutes a large proportion of the protein in serum. This allows the measurement of TP to provide an estimation of serum Ig concentration. The methodology of this procedure is very simple. Blood samples are collected from calves in tubes containing no anticoagulant; serum is separated and then collected. A drop of the serum is then placed on the prism of the refractometer and a reading is recorded.

Optimal survival of calves (n = 3,479) through 16 wks of age occurred when serum TP concentrations were greater than 5.5 g/dl (Tyler et al., 1998). Calves with serum TP concentrations of 5.0 to 5.4 g/dl had a slight increase in relative risk of mortality compared to calves with serum TP concentrations greater than 5.5 g/dl. Calves with serum TP concentrations of $\leq$ 4.4 g/dl had the greatest relative risk of mortality. When a TP concentration of 5.2 g/dl was used as the cut-off point of indicating adequate passive transfer, serum TP had 83% specificity when compared to RID determination of passive transfer in calves (Dawes et al., 2002). This method incorrectly classified 4 calves as adequate passive transfer, 18 calves (n = 119) as FPT. In another study that used 5.5 g/dl as a cut-off point, TP had 80% specificity, sensitivity and accuracy when compared to RID determination of passive transfer in calves (McVicker et al., 2002).

Calloway et al. (2002) evaluated 3 different refractometers for detection of FPT transfer in calves using 5 different TP end-points (4.8, 5.0, 5.2, 5.5 and 6.0 g/dl). Specificity of all three refractometers was greater than 80% when TP end-points were 4.8, 5.0 or 5.2 g/dl. Specificity of all three refractometers decreased (0.56, 0.58 and 0.78, respectively) as TP end-point increased to 5.5 and 6.0 g/dl. Sensitivity increased as the end-point was increased for all three refractometers, and was 0.96 at 6.0 mg/dl. Proportion of calves correctly classified was greatest (0.86 to 0.87) when TP end-points of 5.0 and 5.2 were used. It was further estimated that serum TP concentrations of 5.0 and 5.5 g/dl were equivalent to serum IgG concentrations of 890 and 1,340 mg/dl, respectively.

Regardless of technique utilized to determine IgG status, only 2.1% of U.S. dairy operations routinely measure passive transfer status of calves (USDA, 2007). The goal of any calf monitoring program is not to predict the health fate of each calf, but to monitor the success of passive transfer on an individual farm and provide additional support to FPT calves. It can also provide a method to evaluate the MC management program. The health status of a calf can be altered by the environment and management that the calf is exposed to, regardless of serum IgG or TP concentration at 24 h of age. Current industry standards for 24 h serum IgG concentration or TP level should be used as a guideline, however farms with different levels of calf management may need to adjust their baseline. Developing a cost effective, rapid, easy to use calf-side test would greatly benefit the dairy producer.

Use of Caprylic Acid to Purify Immunoglobulins

While the use of caprylic acid **(CA)** has not been previously used to determine IgG content of bovine MC or calf blood, CA has been used to purify Ig from serum/plasma samples (Bergmann-Leitner et al., 2009; Perosa et al., 1990; Tomita et al., 1995) non-lactating mammary secretions (Guidry and O'Brien, 1996) and may be a potential method to adapt to bovine MC. Caprylic acid precipitates out contaminants and non-Ig proteins while the IgG molecules stay in solution (Grodski and Berenstein, 2010). Caprylic acid also acts to inactivate viruses (Mpandi et al., 2007, Parkkinen, 2005) and is often used to fraction out IgG to be used for intravenous Ig treatments. Caprylic acid is also bactericidal against the major bovine mastitis pathogens *Strep. agalactiae, Strep. dysgalactiae, Strep. uberis, Staph. aureus and E. coli* in milk (Nair et al., 2005).

If CA can be adapted to MC or blood as a method to precipitate out IgG, the supernatant could be analyzed similar to TP using a refractometer. This would provide producers with a simple, rapid and inexpensive method to directly measure IgG concentration in MC and blood on the farm.

Methods to Estimate Intestinal Transport of Nutrients

Determining the IgG status of the calf is an important tool to producers to determine if a calf has achieved adequate passive transfer, however, it is just as important to determine intestinal transport kinetics of nutrients in MC, CR and CS. Knowing how the amount of a nutrient absorbed into circulation and how it is transported across the intestinal epithelium allows for the modification of formulas to better meet the animal's needs. It also allows researchers to determine what impacts absorption of specific nutrients. There have been various methods used to determine intestinal transport *in vivo, in vitro* and *ex vivo,* with methodologies being altered as understanding of transport systems has increased as well as to meet the goals of a specific physiological state.

Internal Intestinal Loops

The earliest studies aimed at determining intestinal transport involved studies of intestinal segments in anesthetized animals (Thiry, 1864; Kimmich, 1975). Early intestinal studies involved surgical preparation of a blind sac of intestine joined to the abdominal wall and open to the animal's exterior. This procedure was eventually modified so that both ends of the chosen intestinal segment were joined to the abdominal wall, thus allowing substances to be introduced at one end of the segment and collected at the other. These early studies led to the development of internal intestinal loops techniques.

Fisher and Parsons (1949) developed a technique for circulating an oxygenated saline solution on both sides of an intestinal loop maintained *in vitro* and were successful in demonstrating that the transport capability of the intestinal segment could be maintained outside the animal. Several modifications to the Fisher and Parsons technique were developed, most notably those introduced by Wiseman (1953) and Darlington and Quastel (1953). The intestinal loop procedure allows for the study of uptake and transport of nutrient by intestinal cells in the live animal. The animal of interest is given anesthesia and intestinal loops can be created in the desired section of the intestines. The loops can be inoculated with labeled latex, dextran, IgG or other metabolite. The intestinal loops are then repositioned inside the animal and the incision site is closed with sutures. After a specific exposure time the animal is euthanized, and the intestinal loops can be removed for histological and immunohistochemical analyses.

Transport mechanisms studied

Internal intestinal loops have been used to study differences in particle uptake between the distal ileal Peyer's patch and jejunal Peyer's patch in calves, effect of particle size on uptake, and effect of age on uptake (Lwin et al., 2009), as well as intestinal Ig secretion in pre-ruminant calves (Porter et al., 1972)

Strengths & weaknesses of technique

Keeping the animal alive and anesthetized while the intestinal loops are exposed to the metabolite of interest, allows for normal metabolic processes to continue. Blood samples can be taken to determine if blood constituents are altered by the incubated metabolite. In addition to determining transport kinetics of metabolites, this method can also be used to determine intestinal secretions under normal or altered conditions. A weakness of this technique is that it may be deemed unnecessary or inhumane by current university animal care and use committees as well as animal welfare advocates.

Everted Intestinal Rings and Sacs

The methodology for the everted intestinal sac technique was originally developed with isolated small intestine from the rat and golden hamster and was first utilized to measure respiration and glycolysis during periods of active transference of substances across the intestinal wall (Wilson and Wiseman, 1954). The everted sac technique of Wilson and Wiseman (1954) allowed tissue samples to be obtained for assay of accumulated materials. This procedure still required one segment for each sample, but the segments were much smaller than those used in perfusion techniques, and several sacs could be prepared from one intestinal region. Data obtained from the Wilson and Wiseman techniques as well as the technique modified by Agar et al. (1954) helped establish that intestinal tissue itself accumulates solutes against a concentration gradient. These conclusions led researchers to develop techniques that could determine molecular mechanisms of absorption intestinal epithelial cells.

For everted intestinal rings and sacs, the animal is euthanized and the intestines are removed from the animal, flushed with a 0.9% sodium chloride solution, removed from the mesentery and can be stored in Krebs-Ringer buffer bicarbonate solution under an atmosphere of 95% O_2 and 5% CO_2 (Philips et al., 1976, Lecce, 1966). For everted intestinal rings the jejunum is everted with a glass rod. The everted jejunum is washed in a balanced salt solution and sliced into 5-mm pieces. A gut slice is placed in a 30 mL beaker containing

5 mL of the incubation medium. After the pre-determined incubation time the gut ring is removed (Lecce, 1966). For everted intestinal sacs, a stainless steel rod is used to push the ileal end of the gut lumen until it appears at the duodenal opening of the intestine. The everted intestine is placed in glucose and saline solution until it is ready to be cut. A determined length of intestine is tied off and one ligature is placed loosely around the other end. A blunt needle attached to a 1 ml syringe is introduced into the intestinal lumen and the loose ligature is pulled tightly over the needle. The appropriate fluid and O_2 bubble can then be injected into the sac as the needle is withdrawn and the ligature is tied (Wilson and Wiseman, 1954). This method has been modified for different species and different areas of research. A miniature everted sleeve technique has more recently been developed to study solute uptake in biopsy specimens of human and animal mucosa (Stelzner et al., 2001).

Transport mechanisms studied

Everted intestinal sacs and rings have been utilized to study nutrient absorption in numerous species. Lecce (1965/66) devised a method utilizing porcine intestinal epithelium to determine if γ-globulin was absorbed by passive diffusion. This experiment showed that uptake of γ-globulin required oxygen and sodium and was reversibly inhibited by metabolic antagonists. Lecce (1965/66) concluded that the transfer of macromolecules was not by passive diffusion but was an energy-coupled reaction.

Lecce (1972) determined the capacity of the neonatal intestinal epithelium to absorb proteins. He utilized gut slices that were incubated for 1 h and then examined for fluorescent globules. At the level of the intestinal epithelium the mouse is no more selective than the piglet and macromolecules of divergent character were all internalized within the gut cell. Everted intestinal sacs have been utilized to determine where in the small intestine AA uptake occurs and that absorption patterns of AA may be different with an amino acid mixture as compared to a single amino acid (Philips, 1976).

Strengths & weaknesses of technique

Strengths of this technique include the researcher having the ability to determine specific nutrient transport mechanisms without the interference of other compounds. These techniques have limitations in that cellular metabolism is still occurring, and that the researcher cannot regulate the intracellular substrate and electrolyte concentration gradients (Wilson and Webb, 1990).

Membrane Vesicle Technique:

The use of isolated membrane vesicles as model for transport studies was developed in the early 1970's. Under appropriate homogenization conditions, the isolated membranes form vesicles so that the membrane separates two aqueous phases. Measuring the solute flux into or out of these vesicles allows for transport properties to be determined (Hopfer, 1977)

Systems studied

Isolated brush border (BB) and basolateral (BL) membrane vesicles have been used to evaluate the transport properties of the small intestine and kidney of several non-ruminant species. The isolated vesicle system has been utilized to obtain information on mechanisms of solute translocation across plasma membranes, coupling of Na^+ and non-electrolyte transport in the basolateral membrane, kinetics of non-electrolyte transport under controlled cis- and trans-situations and the analysis of transport changes associated with different physiological or pathological states (Hopfer, 1977).

Isolation of intestinal BB and BL membrane from ruminants was first reported in 1990 by Wilson and Webb, who were able to demonstrate that the BB and BL membrane vesicles were suitable tools for characterizing the transport properties of the bovine intestinal enterocyte. The BB membrane vesicles can be used to evaluate the transport properties regulating entry of nutrients into the enterocyte. The BL membrane vesicle can be utilized to monitor transport properties leaving the enterocyte and entering the circulation (Wilson and Webb, 1990).

Strengths & Weakness of Technique

The absence of cellular metabolism in BB and BL membrane vesicles allows for the characterization of transport properties of metabolizable substrates (Hopfer, 1977; Wilson and Webb, 1990). Isolated BB and BL membrane preparations are suitable models for evaluating nutrient transport properties of the bovine small intestine, and many more transport experiments per animal can occur as compared to other in vitro techniques (Wilson and Webb, 1990).

Ussing Chambers:

The Ussing chamber was originally designed to study vectorial ion transport through frog skin (Ussing and Zerahn, 1951). Since its introduction by Hans Ussing, it has been utilized in a broad range of applications. The Ussing chamber is an *ex vivo* technique in which tissue is collected and mounted between two buffer containing reservoirs (the luminal and serosal chambers; Awati et al., 2009).This technique allows for the measurement of actively transported ions, nutrients and drugs as well as determining the permeability of tissues, thus permitting the study of absorption of compounds across the mounted tissue (Awati et al., 2009; Clarke, 2009). Ussing chambers have been used as an *ex vivo* model to investigate intestinal nutrient absorption in rats (Moazed and Hiebert, 2007) and pigs (Boudry, 2005). Ussing chambers have been utilized to study nearly every epithelium in the animal body, including the reproductive tract, exocrine/endocrine ducts, intestine, airways, eyes and the choroid plexus (Clarke, 2009).

Methodology

For intestinal studies, the animal is euthanized and segments of the intestine are harvested. The mucosa is stripped from the seromuscular layer and placed in an aerated buffer solution for transport to the laboratory. The intestinal segment is then removed from the buffer, opened and oriented as a flat sheet to separate the two halves of the chamber. The intestinal tissue is situated vertically such that the mucosal membrane is facing one chamber and the serosal membrane is facing the other chamber. A CO_2/HCO^-_3 buffered Ringer solution is used as a bathing solution, 10 mM of glucose is added to the serosal side and 10 mM of mannitol is added to the mucosal side to osmotically balance the chambers. Oxygen (95%) and carbon dioxide (5%) tension are maintained in the physiological buffer by injection ports.

Electrodes can be connected to allow the measurement of the electrical resistance of the tissue, or its inverse, the conductance. This is considered to reflect the function of the tight junctions between epithelial cells, or the paracellular permeability of the tissue (Boudry, 2005). Multiple chambers can allow for the study of nutrient transport across multiple locations of the G.I.T. of a single animal.

Systems studied

Ussing Chambers have been used to study the intestinal permeability of glucose (Ducroc et al., 2007), heparin (Moazed and Hiebert, 2007), oligonucleotides (Wu-Pong et al., 1999), drugs (Lampen et al., 1998; Boudry, 2005) ions (Holtug et al., 1996) and amino acids (Awati et al., 2009 and Grondahl and Skadhauge, 1997). Ussing chambers have been used in animal agriculture to study the impact of weaning on gastrointestinal health in piglets (Moeser et al., 2007) and the permeability of intestines to bacteria and infectious agents (McKie et al., 1999; Smith et al., 1992). Rat intestine has been utilized to determine bacterial passage across ileal mucosal segments (Go et al., 1995). Increased permeability to bacteria at the mucosal level contributes to the bacterial translocation seen in endotoxemia. Miyakawa and Uzal (2005) utilized tissue from the ovine ileum and colon to determine changes induced by α toxin. When α toxin was added to the luminal side water flux significantly decreased after 10 min, and then recovered to baseline values at 20 min. Results indicated that α toxin could induce morphologic and physiologic changes in water transport in the ileum and colon. Ussing chambers have also been utilized to determine differences in ion transport in intestinal tissue from normal neonatal mice and neonatal mice with cystic fibrosis (Grubb, 1999).

Strengths & Limitations

The primary strength of the Ussing chamber technique is that is provides a short term organ culture method that enables precise measurement of electrical and transport parameters of intact, polarized intestinal epithelium (Clarke, 2009). The tissue is maintained in an environment that is as close to normal as possible, allowing for the study of permeability of the tissue to various molecules.

While Ussing chambers are beneficial to determine transport, they do have limitations. Because they are an in vitro technique, the excision of the tissue from the animal suppresses the action of any endocrine factors (Boudry, 2005; Clarke, 2009). When using the Ussing chamber as a technique to evaluate the effect of substances or feed additives the compounds being tested may not be in the same form of what would normally reach the intestines of an intact animal. Another limitation is the viability of the tissue once removed from the animal (Boudry, 2005). It is recommended to limit studies to 2 to 3 h to minimize viability problems.

Conclusion

Models that require fewer animal numbers then a large field trial can be used to estimate intestinal tissue permeability and transport mechanisms of various metabolites. They provide researchers with a better understanding of intestinal absorption and changes in absorption when other metabolites or toxins are present and during changes in physiological state of the animal. As more models are validated, researchers will be able to alter feedstuffs to make them more available to the animal. It will also allow for greater understanding of transport mechanisms as well as what alters absorption of select nutrients in specific physiological situations, thus allowing nutritionists to more accurately provide a balanced ration. In regards to MC and the neonatal calf, a model that can provide information on transport mechanisms will allow for the formulation of CR that are more readily absorbed by the neonate, thus providing more effective passive transfer of antibodies.

Challenges with these techniques do exist and must be recognized. It also must be realized that while an additive to a feed product might increase absorption, other metabolic processes that may potentially be altered are unknown. A transport mechanism may also be altered by the physical state of the feedstuff/nutrient as well as the physiological state of the animal.

Overall Conclusion

Neonatal calves face numerous challenges during the first hours of life. They must receive colostral IgG prior to gut closure to assure adequate passive transfer – a phenomenon that is often not measured until after gut closure if at all.

Adapting CA purification to rapidly determine IgG concentration in both MC and serum would be of great benefit to the dairy industry. Producers would be able to more accurately identify high quality MC to feed calves, as well as identifying MC that should be discarded. The ability to rapidly determine calf IgG status would allow producers to identify calves that have obtained adequate passive transfer, as well as calves have not. Due to the fact that CA precipitation and refractometry is a rapid test, calves with FPT could be identified prior to gut closure, thus allowing the producer to provide additional MC or CR to the calf.

The validation of the Ussing chamber technique to determine IgG uptake in the neonatal bovine intestine would allow more research to be done with MC, CR and other milk products. It would allow researchers to look at specific additives, toxins and bacteria that may alter absorption of IgG without having to run a larger field trial. This will provide a direct measurement of compounds being transported across the intestine, while eliminating potential confounding factors that a field trial could introduce.

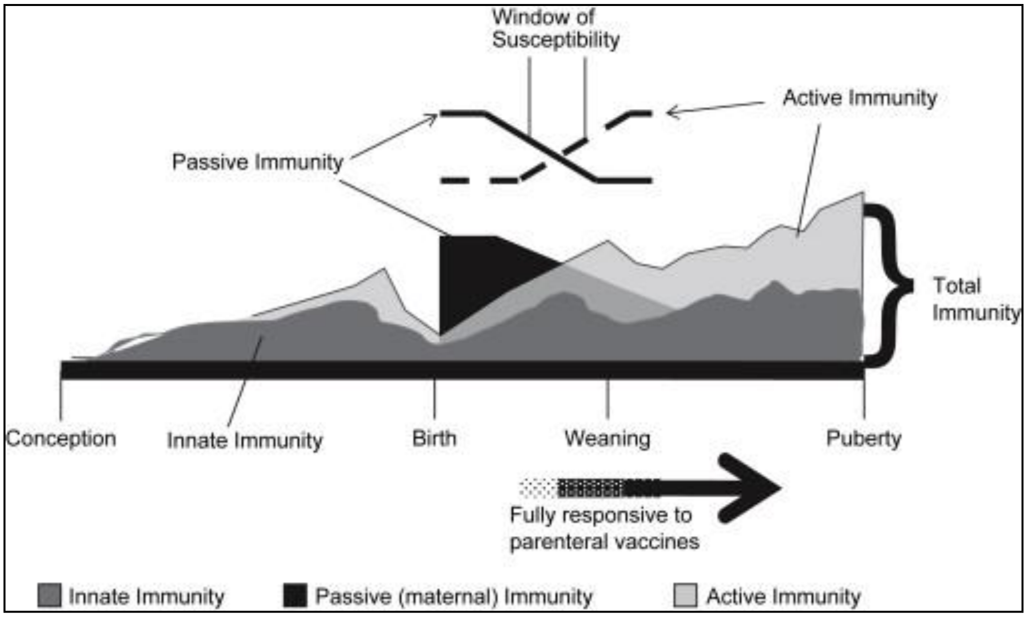


Figure 1: Development of the immune response in the calf: from conception to puberty.

Adapted from Chase et al., 2008.

Decreased native defense mechanisms

- ↓Complement activity
- ↓Neutrophil and macrophage activity
- ↓Interferon production
- ↓Natural killer cell function
- ↓Dendritic cells

Decreased acquired immune mechanisms

- Decreased lymphocyte responsiveness
- Neonates have TH_2 response
- ↓Major histocompatibility complex II: ↓antigen presented to T cells
- Born with no memory T or B cells
- Antibody production ↓ CD40 ↓CD40L B-cell differentiation
- Agammaglobulinemic: must obtain antibody from the mother through colostrum

Figure 2. Immune status of the neonatal calf (Adapted from Chase et al., 2008)

Table 1. Physical characteristics and composition of colostrum and whole milk

Item	Colostrum (no. of postpartum milking) 1	2	3	4	5	Milk
pH	6.32	6.32	6.33	6.34	6.33	6.5
Total solids (%)	23.9	17.9	14.1	13.9	13.6	13.9
Total fat (%)	6.7	5.4	3.9	4.4	4.3	4.0
Solids not fat (%)	16.7	12.2	9.8	9.4	9.5	8.8
Total protein (%)	14	8.4	5.1	4.2	4.1	3.1
Casein (%)	4.8	4.3	3.8	3.2	2.9	2.5
Immunoglobulins(%)	6.0	4.2	2.4	…	…	0.09
Lactose (%)	2.7	3.9	4.4	4.6	4.7	5.0
Ash (%)	1.11	0.95	0.87	0.82	0.81	0.74
Ca (%)	0.26	0.15	0.15	0.15	0.15	0.13
Mg (%)	0.04	0.01	0.01	0.01	0.01	0.01
K (%)	0.14	0.13	0.14	0.15	0.14	0.15
Na (%)	0.07	0.05	0.05	0.05	0.05	0.04
Cl (%)	0.12	0.1	0.1	0.1	0.1	0.07
Zn (mg/100 ml)	1.22	…	0.62	…	0.41	0.3
Mn (mg/100 ml)	0.02	…	0.01	…	0.01	0.004
Fe (mg/100 ml)	0.2	…	…	…	…	0.05
Cu (mg/100ml)	0.06	…	…	…	…	0.01
Co (mg/100ml)	0.5	…	…	…	…	0.1
Vitamin A (µg/100 ml)	295	190	113	76	74	34
Vitamin E (µg/g fat	84	76	56	44	31	15
Riboflavin	4.83	2.71	1.85	1.8	1.76	1.47
Choline	0.7	0.34	0.23	0.19	0.16	0.13

*Adapted from Akers, 2002; Foley and Otterby, 1978

CHAPTER TWO

A RAPID METHOD TO DETERMINE COLOSTRUM QUALITY

ABSTRACT

Caprylic acid **(CA)** has been utilized to fractionate colostral IgG for further laboratory purification and analysis. The objective of this study was to develop a rapid, cow-side test for determining colostrum IgG concentration using CA fractionation followed by refractometry of the IgG-rich supernatant. Frozen colostrum samples (n = 85) obtained from Holstein cattle, were warmed to room temperature in a water bath and treated with varying concentrations of CA and acetic acid **(AcO)**. Samples were then centrifuged or allowed to incubate for an allotted time to precipitate non-IgG proteins. Supernatant liquid was then analyzed with a digital refractometer (SPER Scientific, model 300034) to determine refractive index **(nD)**. The nD of Ig-rich fraction was compared to total colostral IgG concentration determined by radial immunodiffusion **(RID;** Triple J Farms, Bellingham, WA). The nD of supernatant was positively correlated (r = 0.96) to RID when 1 ml of colostrum was added to a tube containing 75 µl CA and 1.5 ml 0.06 M AcO, shaken for 10 s and not centrifuged. Refractive index was measured within 1 min of addition of CA. Decreasing AcO to 1 ml or increasing AcO to 2 ml decreased the correlation (r = 0.73 and r = 0.63, respectively) between nD and IgG. For centrifuged samples, altering the incubation time prior to centrifugation from 30 min to 0, 10 or 20 min numerically increased correlation (r = 0.82 to 0.87, 0.85 and 0.87, respectively), but these were not statistically different. When the centrifuge step was removed, and samples incubated for 1 min the nD was highly

correlated (r = 0.96) with RID-determined IgG concentration; however visible separation of supernatant and precipitate did not occur in samples with IgG concentrations > 20 mg/ml until after 10 min. Total protein was measured on a subset of 45 samples and weakly correlated (r = 0.41) with IgG; this suggests that total protein is a poor method to determine colostral IgG concentration. These results indicate that a simple procedure requiring only CA, AcO and a refractometer may rapidly and effectively estimate colostral IgG concentration in bovine colostrum.

Key words: colostrum, analysis, IgG, refractometer

INTRODUCTION

Newborn animals of many species rely on the ingestion of adequate amounts of maternal colostrum **(MC)** to provide them with the nutrients, antibodies, and bioactive peptides. Immunoglobulins **(Ig)** cannot cross the placental structure of cattle; therefore calves are born aggamaglobulinemic with no measurable circulating IgG or IgM. High quality MC, defined as having an IgG concentration greater than 50 mg/ml (McGuirk and Collins, 2004), fed within the first hours of life provides the calf with sufficient amounts of IgG to provide passive immunity for the first 30 to 90 d of life (Guy et al., 1994) until the immune system of the calf is better equipped to respond to pathogens on its own. It is recommended that a minimum of 100 g of IgG be fed to newborn calves within the first 24 h of life (Davis and Drakley, 1998). If MC containing 50 mg/ml is available, the calf should be fed a minimum of 2 L to obtain 100 g IgG. For producers to feed adequate amounts of IgG, the IgG concentration of MC must be determined. Unfortunately there are few tools available to dairy producers that are able to rapidly assess MC quality on farm. Currently only 13% of US dairy operations evaluate MC IgG concentration prior to feeding (NAHMS, 2007). Farms that have a herd size of 500 head or more are more likely to evaluate MC quality (45.2%) compared to farms with less than 100 head (7.6%). The most common methods of MC measurement on farms that determine MC quality are the use of a colostrometer (43.7% of operations) and visual appearance (41.6% of operations; NAHMS, 2007).

The colostrometer was introduced as a practical field tool for measuring IgG concentration in bovine MC based on the linear relationship between colostral specific gravity and IgG concentration (Fleenor and Stott, 1980). The colostrometer is calibrated in IgG concentration at intervals of 5 mg/ml from 0 to 180 mg/ml, displayed with 3 color coded quality regions: poor (red) less than 22 mg/ml, moderate (yellow) 22 to 50 mg/ml and excellent (green) greater than 50 mg/ml. The colostrometer is not an analytical technique, but rather a method that can be utilized to estimate relative quality of MC. While colostrometers have allowed producers to identify poor or quality MC, they are often inaccurate (Morin et al., 2001). Colostrometers often overestimate IgG concentration (Mechor et al., 1992) or are utilized improperly (Mechor et al., 1991). The quality readings on the colostrometer are based on the specific gravity of normal milk and provide an estimate of relative quality, not actual IgG quantity (Fleenor and Stott, 1980; Quigley et al., 1994). Colostral specific gravity can be affected by breed, month of calving and parity (Morin et al., 2001).

A second tool available to producers is the refractometer. Liquid solutions refract light, and refractometers use this property to estimate total protein **(TP)** in that solution (Chavatte et al., 1998). A refractometer can provide a reasonable estimation (R^2 = 0.76) of IgG concentration when MC is measured at 20^0C (Mechor et al., 1992). Refractometers have been used to determine the IgG content in colostral whey (Molla 1980), and TP in whole

MC. Optical and digital Brix refractometers have a correlation coefficient between 0.71 and 0.74 when compared to IgG concentration determined by radial immunodiffusion **(RID)** for both fresh and frozen MC (Bielmann, 2010). Refractometers are readily available tools that can be utilized, on-farm, to measure the TP content in MC; however the IgG concentration is of more value to the dairy farmer than the TP content. Removal of the non-antibody proteins from MC would significantly improve the value of the refractometer as a method to determine MC quality. Developing a method to accurately determine colostrum IgG content using a refractometer would be of great benefit to dairy producers.

Aside from colostrometers and refractometers, there are also laboratory techniques that allow for accurate quantification of IgG concentration, however, these methods can require up to 48 h to obtain a value for IgG concentration, well beyond the time of gut closure. These techniques are often not readily available to producers and are often expensive. Guidry and O'Brien (1996) utilized CA to precipitate non-IgG from bovine lacteal secretions, leaving the IgG in the supernatant for analysis. This method diluted the secretions from non-lactating mammary glands (1:4 vol/vol) with acetate buffer (60 mM; pH 4), and adjusted the pH to 4.5 with 1 M Tris base. Caprylic acid (30 µl/ml of diluted sample) was then added by drop with mixing. Samples were mixed for 30 min, and centrifuged for 30 min. After centrifugation, the supernatant was adjusted to a pH of 7.4 with Tris base, and analyzed for IgG concentration using an enzyme-linked immunosorbent assay. The CA lowered non-specific binding of IgM, and more accurately measured specific activity of IgG (Guidry and O'Brien, 1996).

It is hypothesized that the CA precipitation method may be simplified and applied to bovine MC to rapidly and accurately determine colostral IgG concentration. The objectives of this study are to 1) modify the Guidry and O'Brien (1996) technique utilized to measure IgG concentration and adapt it to bovine MC and 2) validate the results obtained with a refractometer with the results obtained utilizing an RID assay.

MATERIALS AND METHODS

Frozen bovine MC samples (n = 85) from Holstein cattle from a California dairy were provided by APC, inc. (Ankeny, IA). Colostrum samples were thawed in a warm water bath and brought up to room temperature (20^{o} C). All samples were analyzed within 4 h of thawing.

RID Analysis

One ml of MC was added to 3 ml of distilled water and mixed well. Five µL of diluted colostrum solution was added to each well of a bovine IgG RID test plate (Triple J Farms, Bellingham, WA). Radial immunodiffusion plates were incubated for 24 h and then the diameter of precipitin ring was measured. IgG concentrations of MC samples were determined by comparing the diameter of the precipitation ring with a standard curve generated by assaying the internal standards of each kit.

Total Protein

One ml of MC was added to 2 ml of distilled water and mixed well. A drop of diluted MC (~50 µl) was placed on a refractometer prism (SPER SCIENTIFIC model 300034; Scottsdale, AZ) and a brix and nD reading was recorded for each sample. The digital refractometer determines the brix or nD of the liquid being analyzed by shining a light through the sample in the well, measuring the index of refraction and presenting the reading in brix or nD units on a digital scale. The nD is the refractive index of a solution, measured at the wavelength of the sodium D line (589.3 nm) at 20 °C. The Brix value can be obtained from the polynomial fit to the International Commision for Uniform Methods of sugar analysis (2009) table: brix = (((((11758.74*nD -88885.21)*nD + 270177.93)*nD - 413145.80)*nD + 318417.95)*nD -99127.4536).

Trial # 1

Trial one was designed to determine if the original protocol designed for secretions from a non-lactating gland, as described by Guidry and O'Brien (1996), was viable when applied to bovine MC. One mL of MC was added to a 12 x 75 mm disposable culture tube, with a designated treatment (Table 1). Colostrum, AcO and CA were mixed well (10 sec) and then incubated for the allotted time. Samples were shaken every 5 min prior to centrifugation. Samples not centrifuged were shaken (10 sec) only upon addition of MC to the tube. The centrifuged samples were centrifuged at 3,000 x g for 10 min. One drop of supernant (~50 µL) was placed on a refractometer prism (SPER SCIENTIFIC model 300034; Scottsdale, AZ) and a brix and nD reading for each sample was recorded.

Trial # 2

Trial two focused on determining the optimal amount of CA and AcO utilized when samples were not centrifuged. One ml of MC was added to a tube containing varying amounts of CA and AcO (Table 2). Once the MC was added to the tube containing the acids, samples were mixed (10 sec) and allowed to incubate for 30 min. After 30 min, the supernatant was removed and analyzed by refractometry.

Trial # 3

Trial three focused on determining the optimal incubation time for the procedure when 75 µl of CA and 1.5 ml of AcO were utilized, and none of the samples were centrifuged. One ml of MC was added to a tube containing the acids and shaken for 10 sec. Samples were then allowed to incubate for 0, 1, 3, 5, 10, 15, 20, 25, 30, 45 or 60 min prior to the removal and analysis of the supernatant by refractometry.

Statistical Analysis

Refractive index (brix and nD) and RID obtained IgG concentration were evaluated by linear regression. A Pearson correlation coefficient was calculated using the PROC CORR procedures of SAS (SAS 9.2, SAS Institute Inc., Cary, NC). The Fisher exact test was performed to determine statistical differences between different treatments.

RESULTS & DISCUSSION

Trial #1

Mean IgG concentration of MC samples (n=35) was 79.0 mg/ml (SD = 31.8) with a range from 25.80 to 128.30 mg/ml. Treatments with no AcO or reduced incubation times (nD) resulted in the weakest relationship with RID obtained IgG concentrations (Table 3). Addition of acids prior to addition of MC had the highest R^2 with RID (Figure 1). Treatment # 8 had a moderate relationship and treatment #6 had a weak relationship with RID obtained

IgG concentration. Adding MC prior to AcO and CA and not centrifuging the sample resulted in the strongest relationship between nD and RID. Treatments 2 and 3 had strong relationships between the %Brix reading and RID, but not with nD reading. A moderate relationship was observed for treatment 1 between nD and RID obtained IgG concentration.

Adding MC to a tub containing CA and strengthened the relationship between nD and RID obtained IgG concentration for original protocol (r = 0.55 and 0.66, respectively) and the protocol with a reduced incubation time (r = 0.30 to 0.75). Removing AcO from the protocol weakened the relationship between nD and RID obtained IgG concentration. This could be due to the inclusion of fat in the supernatant, potentially altering the refractive properties of the supernatant. Based on the results of trial #1 it was determined that having CA and AcO in the tubes prior to addition of MC improved the relationship between nD and RID obtained IgG concentration. It was also decided to further investigate protocol that removed the centrifuge step altogether.

Trial #2

Mean IgG concentration for MC used in trial two (n=10) was 59.44 mg/ml (SD=25.04) with a range of 23.20 to 110.80 mg/ml. Protocols that added either 1 or 1.5 ml of AcO had correlation coefficients greater than 0.70 with the RID analysis of IgG, regardless of CA amount (Figure 2). Conversely, protocols that added 2 ml of AcO had correlations with the RID obtained IgG concentration ranging from 0.31 (P = 0.3695) for the 90 µl CA treatment to 0.65 (P = 0.0390) for the 75 µl CA treatment. The treatment containing 75 µl CA and 1.5 ml of AcO had the strongest correlation (r = 0.80, P = 0.0051) to the IgG concentration determined by the RID analysis of the MC sample.

Trial #3

Mean IgG concentration for MC used in trial three was 58.87 mg/ml (SD = 33.29) with a range from 2 to 116 mg/ml. Sitting times of 1, 3, 5, 10, 15, 20, 30, 45 and 60 minutes prior to analysis of supernatant all provide a correlation coefficient greater than 0.90 (Table 4) while analyzing the sample immediately (NC0) resulted in a weaker relationship between refractive index and IgG concentration. Removing the centrifuge step from this procedure continued to allow for a strong correlation with the IgG concentration, thus allowing the procedure to be simplified. Determining the optimal incubation time prior to analysis of the supernant yielded similar results to those from altering the incubation time with the centrifuge step. There were no improvements in the accuracy of the protocols when samples were allowed to incubate for a minimum of one min, or a maximum of 60 min. For most samples that were not centrifuged, visual separation did not occur until 10 min. Samples that immediately separated (1 and 3 minutes) had %Brix readings less than 4.0, nD reading less than 1.33895 and RID values less than 20 mg of IgG/ml.

Total protein (brix and nD) was measured on 45 samples. Mean IgG concentration for sample utilized was 80.05 mg/ml (SD = 31.49) with a range from 25.80 to 140.00 mg/ml. The relationship between nD and RID obtained IgG concentration was weak ($r = 0.41$, $P = 0.006$ for brix and $r = 0.38$, $P = 0.0101$ for nD, respectively). These results suggest that TP is an inadequate method to determine MC quality (Figure 3).

CONCLUSION

Simplifying the procedure described by Guidry and O'Brien (1996) by increasing the amount of both AcO (1.5 ml) and CA (75 μl) and removing the centrifuge step provides a high correlation with the IgG concentration of bovine MC as determined by RID (r = 0.96). Further research should be done to validate this procedure on-farm as well as to determine if the accuracy of this procedure is impacted by breed, parity, nutrient content or bacterial contamination of MC.

A. %Brix B. %nD

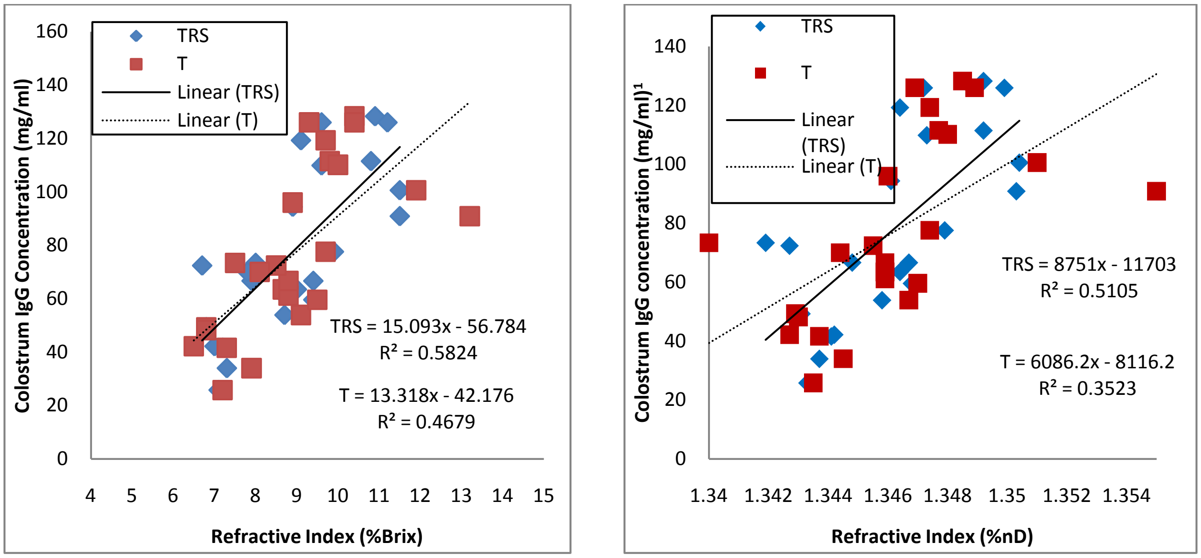
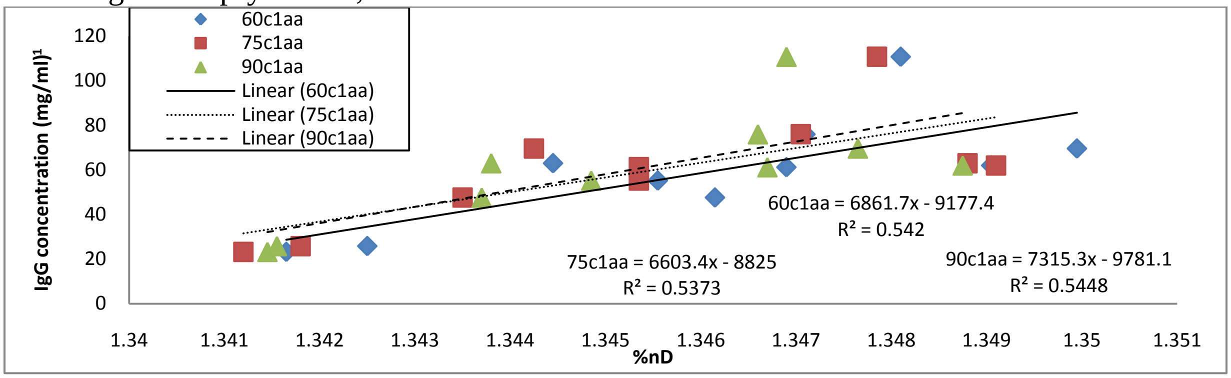
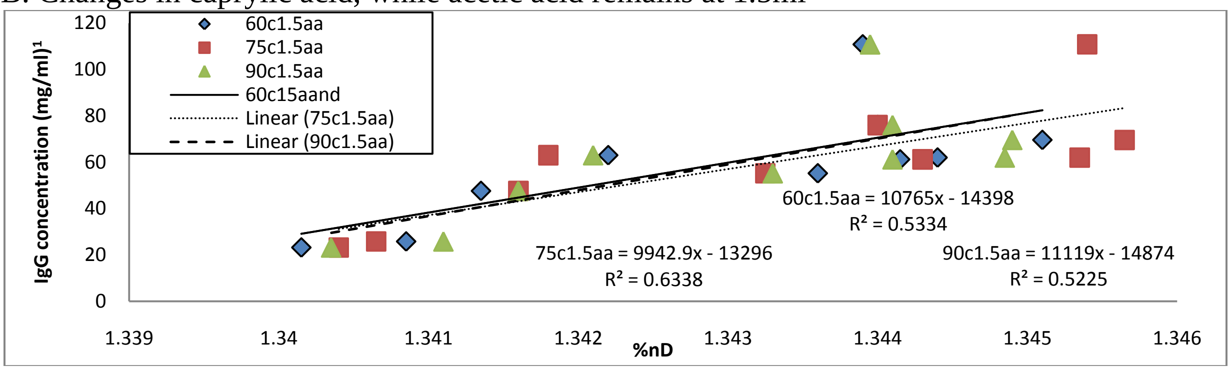


Figure 1 – The %Brix and nD values for treatments T and TRS compared to colostrum IgG concentration obtained by RID analysis. T = acids in tube prior to colostrum, allowed to sit for 30 minutes, and then centrifuged. TRS = Acids in tube prior to colostrum, allowed to sit for 15 minutes prior to centrifugation.

A: Changes in caprylic acid, while acetic acid remains at 1 ml



B. Changes in caprylic acid, while acetic acid remains at 1.5ml



C. Changes in caprylic acid, while acetic acid remains at 2 ml

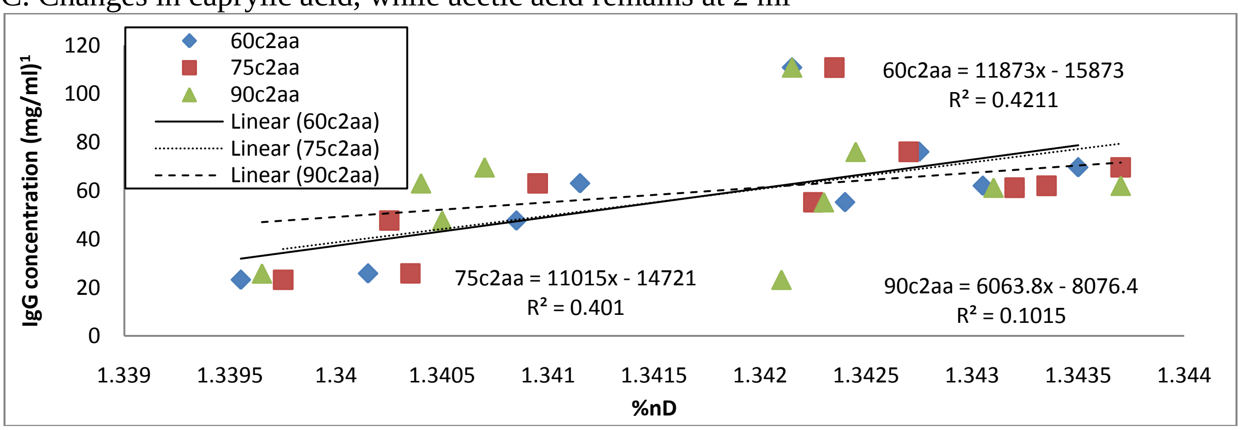


Figure 2: Regression analysis for nD reading compared to IgG concentration for samples with different amounts of caprylic acid and acetic acid. Treatments correspond to amounts of acids in tube prior to addition of colostrum, c = caprylic acid, aa = acetic acid. No samples were centrifuged.

[1]As determined by radial immunodiffusion.

A. %Brix B. %nD

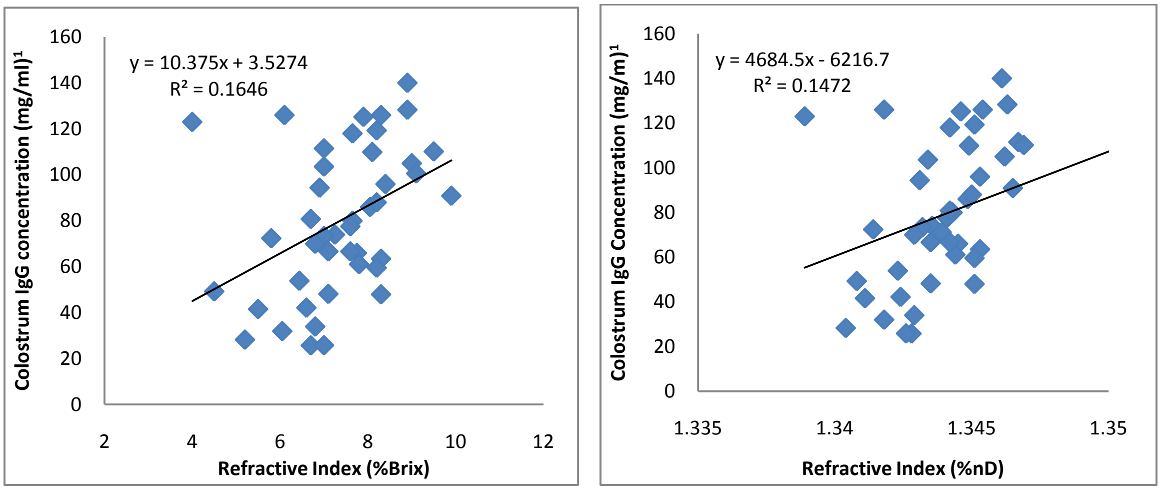


Figure 3: Regression analysis for total protein refractive index compared to IgG concentration of sample.
[1]As determined by radial immunodiffusion.

Table 1. Treatment modifications tested during trial #1

Treatment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
n[2]	28	29	30	16	30	35	29	19
First addition	MC[1]	MC	MC	MC	AcO	CA	AcO	AcO
Second addition	AcO[2]	CA	AcO	AcO	CA	MC	CA	CA
Third addition	CA[3]	none	CA	CA	MC	none	MC	MC
Incubation time (min)	30	30	15	30	30	30	15	30
Centrifugation time (min)	10	10	10	0	10	10	10	0

[1]MC = addition of 1 ml maternal colostrum
[2]AcO = addition of 1 ml 0.06 M acetic acid
[3]CA = addition of 60 µl caprylic acid

Table 2: Acetic and caprylic acid concentrations tested for trial #3

Treatment	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
N[1]	10	10	10	10	10	10	10	10	10
Caprylic acid (µl)	60	60	60	75	75	75	90	90	90
0.06 M Acetic acid (ml)	1	1.5	2	1	1.5	2	1	1.5	2

[1] n = number of observations for individual treatments.

Table 3. Correlation coefficients between RID determined IgG concentration and Brix or nD refractometer reading for Trial 1 treatments

Treatment[1]	n	RID*Brix		RID*nD	
		r	p-value	r	p-value
1	28	0.56	0.0018	0.55	0.0022
2	29	0.72	<0.001	0.25	0.1831
3	30	0.63	0.0002	0.30	0.1116
4	16	0.71	0.0025	0.74	0.0006
5	30	0.71	<0.0001	0.66	<0.0001
6	35	0.48	0.0039	0.49	0.0031
7	29	0.78	<0.0001	0.75	<0.0001
8	19	0.63	0.0039	0.62	0.0050

[1] Treatments are abbreviated O = original protocol, NA = no acetic acids added, RS = reduced sitting time before centrifuge step, NC = no centrifuge step, T = acids were in tube prior to colostrum.

Table 4. Correlation coefficients between RID determined IgG concentration and Brix or nD refractometer readings for trial 4

		RID*Brix		RID*nD	
Treatment[1]	n	r	p-value	r	p-value
NC0	10	0.63	0.049	0.68	0.0293
NC1	30	0.96	<0.0001	0.95	<0.0001
NC3	20	0.96	<0.0001	0.96	<0.0001
NC5	30	0.95	<0.0001	0.95	<0.0001
NC10	30	0.94	<0.0001	0.94	<0.0001
NC15	10	0.95	<0.0001	0.95	<0.0001
NC20	10	0.91	0.0002	0.91	0.0003
NC25	10	0.81	0.0045	0.82	0.003
NC30	10	0.95	<0.0001	0.95	<0.0001
NC45	10	0.95	<0.0001	0.94	<0.0001
NC60	10	0.92	0.0002	0.92	0.0002

[1] Treatments: NC = no centrifuge step followed by the min allowed to sit prior to the analysis of supernatant.

CHAPTER THREE

ON-FARM VALIDATION OF CA TEST AND COMPARISON TO WHOLE REFRACTIVE INDEX AS A METHOD TO ESTIMATE IgG IN BOVINE COLOSTRUM

ABSTRACT

Our objective was to validate a rapid, cow-side method to estimate IgG in bovine maternal colostrum **(MC)** based on caprylic acid **(CA)** fractionation of MC followed by refractometry of the IgG-rich supernatant and to compare this method with refractometry of whole MC. Samples of MC (n = 827) were collected from 67 farms in 12 states. Samples were fresh (not stored; n = 196), previously frozen (n = 490) or refrigerated (n = 152). One ml of MC was added to a tube containing 75 µl CA and 1 mL 0.06 M acetic acid and refractive index **(nD)** of the IgG-rich supernatant was determined using a digital refractometer (SPER Scientific, model 300034). Whole, non-fractionated, MC was analyzed for nD and IgG by radial immunodiffusion. The correlation between nD of CA supernatant and IgG (r = 0.53; $P \leq 0.05$) was low, whereas the correlation between nD of whole MC and IgG was greater (r = 0.73; $P \leq 0.05$). Correlations between nD of whole MC and IgG were similar for Holsteins (r = 0.77; $P \leq 0.05$) and Jerseys (r = 0.80; $P \leq 0.05$). Regression equations were used to estimate the IgG concentration of samples based on the nD of CA supernatant or whole MC. The regression equation created from nD of CA supernatant resulted in 34.3% of samples accurately estimated within 10 mg/ml, where as the regression equation created from the nD of whole MC resulted in 43.8% of samples estimated within 10 mg/ml. These results suggest that breed and lactation do no impact the accuracy of either test

and that refractometry of whole MC provides a more accurate estimation of colostral IgG concentration as compared to the CA test.

Keywords: Colostrum, refractometer, passive transfer, IgG

INTRODUCTION

Due to the placental structure of dairy cattle, immunoglobulin G **(IgG)** cannot cross the placental and calves are born aggamaglobulinemic with no measurable circulating IgG or IgM. Feeding high quality maternal colostrum **(MC)**, greater than 50 mg IgG/ml (Godden, 2008), within the first hours of life is necessary to decrease the risk of failure of passive transfer **(FPT)**, which is classified as having a serum IgG concentration $\leq$ 10 mg/ml at 24 h of age (NAHMS, 1996). Calves with FPT are at a greater risk of mortality and morbidity due to an increased susceptibility to pathogens and subsequent disease (Boyd, 1972; McGuire, 1976). Beam et al. (2009) reported the prevalence of FPT in heifer calves in the US to be 19.2%. There are various factors that can impact FPT in newborn calves.

The primary factors that influence FPT are the amount of quality colostrum fed (Stott and Fellah, 1983; Jaster, 2005) and the time elapsed postpartum prior to feeding (Nocek, 1984). To achieve adequate passive transfer in an average Holstein calf (43 kg), it is recommended to feed a minimum of 100 g of IgG in the first MC feeding (Davis & Drakley, 1998) within the first 1 to 2 h of life and by 6 h at a maximum (Godden, 2008). A key component to a MC management program is identifying quality MC, and then feeding an adequate volume to newborn calves within the first hours of life. Currently only 13% of all U.S. dairy operations evaluate MC quality prior to feeding (NAHMS, 2007). For farms that do measure MC quality, the most common methods were use of a colostrometer and visual appearance (43.7 and 41.6%, respectively) volume of colostrum and other methods comprise the remaining 14.7% (NAHMS, 2007). The quality readings on colostrometers are based on the specific gravity of normal milk and provide an estimate of relative quality, not actual IgG quantity (Fleenor and Stott, 1980). Colostrometer readings are affected by temperature of the MC, as well as total solids **(TS)** content (Morin et al., 2001; Mechor et al., 1993). Using the colostrometer on MC that is cold will lead to readings that are greater then what the actual IgG concentration of the sample. Likewise, samples that are too warm will have readings that are lower than the actual IgG concentration.

Another tool that is available to producers is the refractometer. Refractometers, digital or optical, can be utilized to measure the total protein **(TP)** content in MC and calf serum (Calloway et al., 2002; Moore et al., 2009; Bielmann, 2010). Protein solutions refract light, and refractometers use this property to measure TP in a solution (Chavatte, et al., 1998). Immunoglobulins constitute a large portion of the TP in neonatal calf serum, and non-immunoglobulin protein concentration of calf serum is relatively constant. Measuring TP in calf serum through refractometry can aid in identifying potential FPT calves. Colostral IgG is concentrated 5 – 10 times that of maternal serum (Butler, 1974). Due to IgG making up the majority of MC protein, measuring TP in MC can aid in identifying MC of adequate quality. While knowing the TP content of MC is valuable, the actual IgG concentration is of more value to the dairy farmer than the total protein content, as adjustments in volume of MC fed can be made to assure for adequate intake of IgG.

Caprylic acid **(CA)** has been used to purify Ig from serum for use as a therapeutic agent in naïve animals and humans. It has been documented that CA-ammonium sulphate is one of three optimal IgG purification methods relative to high-level antigen specific recovery and maintaining intact and functional IgG after purification (Bergmann-Leitner et al., 2008). Caprylic acid works to precipitate proteins, leaving the IgG in the supernatant; the combination of CA-ammonium sulphate improves the yield and purification of some proteins (Grodski and Berenstein, 2010). When CA is added to lacteal secretions during the dry period, most proteins are precipitated out and IgG can be more accurately measured in the supernatant fraction by enzyme linked immunosorbant assay (Guidry and O'Brien, 1996). Recent research in our lab has adapted the procedure described by Guidry and O'Brien (1996) to determine IgG content in bovine MC via refractive index **(nD)** with a digital refractomter. A limitation to the laboratory development of the CA test is that data was generated from a small sample set, and all MC samples originated from one dairy farm. The potential impacts of lactation, breed, storage methods, nutrient and bacterial contamination could not be determined.

Preliminary results (Morrill et al., unpublished) suggest that the CA test and refractometry may be a reliable method to rapidly determine bovine MC IgG concentration on-farm. Creating an accurate cow-side test to determine MC quality will benefit dairy producers. The objectives of this study were 1) to validate the lab results of the CA test with an on farm trial, 2) determine if this method is the impacted by breed, parity, storage method, nutrient composition and bacterial contamination and 3) compare the CA test to refractometry of whole MC and determine if the precipitation step increases the accuracy.

MATERIALS & METHODS

Colostrum Sample Collection

Eight hundred ninety one MC samples were collected from a 67 farms across 12 states between June and October, 2010 (Table 1). Samples represented a snapshot of MC available at the time when the farm was visited. Samples were collected from Holsteins, Jersey, crossbred and unidentified breed of dairy cattle. Three samples of MC were collected from each individual cow or pool of MC for analysis of nD of whole MC, analysis of nD by the CA test, IgG concentration by RID (analyzed at Iowa State University; Ames, IA), fat, protein, lactose, other solids (OS), TS, milk urea nitrogen (MUN), somatic cell count (SCC), total plate count (TPC) and coliform counts (analyzed by DHI Laboratory; Dubuque, IA). Samples to be analyzed for IgG concentration, nutrient and bacterial composition were frozen, placed on dry ice and shipped to the respective laboratory. Eight hundred and twenty seven MC samples from the initial dataset were utilized in this study.

Colostrum Sample Analysis

Colostrometer reading

A bovine colostrometer (Nasco; Fort Atkinson, WI) was placed in a graduated cylinder containing MC. A reading in mg/ml was recorded at the point of bouyancy.

Refractometer reading – whole colostrum

A drop of whole MC (~50 µl) was placed on a refractometer prism (SPER SCIENTIFIC model 300034; Scottsdale, AZ) and a brix and nD reading was recorded for each sample. The digital refractometer determines the brix or nD of the liquid being analyzed by shining a light through the sample in the well, measuring the index of refraction and presenting the reading in %Brix or nD units on a digital scale. The nD is the refractive index of a solution, measured at the wavelength of the sodium D line (589.3 nm) at 20 °C. The %Brix value can be obtained from the polynomial fit to the ICUMSA (2009) table: brix = (((((11758.74*nD -88885.21)*nD + 270177.93)*nD - 413145.80)*nD + 318417.95)*nD - 99127.4536).

Caprylic Acid Test Analysis

One ml of MC was added to a tube containing 1.5 ml 0.06 M acetic acid and 75 µl CA. Once the MC was added, samples were mixed for 10 sec and allowed to sit for 60 sec. After 60 sec the supernatant was removed and analyzed using the digital refractometer to obtain an nD and brix value.

Radial immunodiffusion analysis

Colostrum samples were thawed in a warm water bath and thoroughly mixed prior to RID analysis. One ml of MC was added to 3 ml of distilled water and mixed well. Five µL of diluted colostrum solution was added to each well of a bovine IgG RID test plate (Triple J Farms, Bellingham, WA). Radial immunodiffusion plates were incubated for 24 h and then the diameter of precipitin ring was measured. The diameter of the precipitin ring was compared to a standard curve created by the internal test standards to determine the IgG concentration. All samples were run in duplicate. Samples with a precipitin ring greater than that of the highest internal standard (26.25 mg/ml) were further diluted and re-analyzed.

Samples with a precipitin ring smaller than that of the lowest internal standard (1.84 mg/ml) were re-analyzed in an undiluted form.

Nutrient & bacterial analysis

Fat, protein, lactose, TS and OS were analyzed on a FOSS Milkoscan FT+ and the SCC was analyzed on a FOSS Fossomatic FC. Total plate counts were performed with the Petrifilm Plate Loop Count method as outlined in section 6.030 of the 17th Edition of the Standard Methods for the Examination of Dairy Products. Coliform counts were performed with the Petrifilm Coliform Count Plate Method (SMEDP 7.071).

Statistical Analysis

The Univariate procedure of SAS (SAS Institute Inc., Cary, NC) was used to determine the frequency of observations, as well as to determine outlier samples that would be removed from the data set.

RID measurement of IgG concentration, colostrometer readings, TS, OS, MUN, protein, fat, SCC log, TPC log, coliform and refractive index scores (nD from the CA test and whole MC) were plotted against each other. Descriptive statistics were used to compare the nD scores to the RID, nutrient and bacterial composition. Pearson coefficients of correlation were calculated using the PROC CORR procedure of SAS (SAS Insititue Inc., Cary, NC) to determine the level of relationship between the nD, RID, colostrometer, TS, OS, MUN, protein, fat, SCC log, TPC log and coliform counts.

Using the correlation between the nD measurement from the CA-quick-test or whole MC and IgG concentration an equation was developed to estimate IgG concentration from the nD of the CA test (in the lab and on farm) and whole MC. These estimates will be referred to as LABEST, ONFARMEST and WEST.

$$LABEST = 9576.7 * \%nd - 12806$$

$$ONFARMEST = 4414.3 * \%nd - 5864.6$$

$$WEST = 2975.1 * whole\%nd - 3995.1$$

Using the actual and estimated IgG concentrations the difference was calculated for all three equations (LDIFF, FDIFF, WDIFF).

LDIFF and FDIFF data were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) according to the model:

$$Y = \mu + La_o + B_p + St_q + LaSt_{Pq} + el_{opq}$$

where Y = the dependent variable; μ = the overall mean; La = the fixed effect of o^{th} lactation (1,…7); B = the fixed effect of p^{th} breed (p = Holstein, Jersey, crossbred); St_q = the fixed effect of the q storage (q = fresh, refrigerated, frozen); LaSt = the fixed effect interaction between o^{th} lactation and q^{th} method of storage; and e = the residual error.

Epidemiological diagnostic test characteristics (sensitivity, specificity and predictive values) were calculated to compare the digital refractometer nD and LABEST, ONFARMEST and WEST to the RID gold standard. Sensitivity was defined as the probability of a test result indicative of an inadequate colostral IgG concentration for a sample with an IgG concentration greater than 50 mg/ml. Specificity was defined as the probability of a test result indicative of an adequate colostral IgG concentration for a sample with an IgG concentration less than 50 mg/ml as determined by means of RID. Predictive values were calculated using the prevalence of samples with an RID IgG concentration of greater than or less than 50 mg/ml and an nD reading above or below the cut point, respectively. The Fisher's exact method was used to determine differences across breeds, lactation and storage method.

RESULTS

Descriptive Analysis

A total of 827 MC samples from the original dataset were analyzed for this study. These samples were collected from 67 farms across 12 states. Samples were collected from 494 Holsteins, 87 Jerseys, 7 crossbreds and 239 of unidentified breed. This dataset encompassed 49 first lactations cows, 174 second lactations cows, 98 third lactations cows, 38 cows in their fourth or greater lactation and 468 samples from unidentified lactations. The majority of the samples represent the first milking after calving, and were frozen for storage prior to feeding.

The IgG concentration determined by RID, CA test nD, whole MC nD, estimates of IgG concentration from the CA test and whole MC analysis are presented in Table 2. The IgG concentration of samples ranged from less than 1.84 mg/ml to 200.18 mg/ml (Figure 1). The overall mean IgG concentration of the samples analyzed was 69.34 mg/ml (SD = 32.8). The IgG concentration increased ($P \leq 0.05$) with parity (42.4, 68.6, 95.9 mg/ml in 1st, 2nd, and 3rd and later lactations, respectively). No differences in IgG were observed due to breed or storage methods. Refractive index of CA supernatant ranged from 1.3374 to 1.3612 with a mean of 1.34424 (SD = 0.00395), while nD of whole MC ranged from 1.3425 to 1.3912 with a mean of 1.36604 (SD = 0.00804). No differences in CA nD or whole MC nD were observed due to breed or lactation; however frozen samples had a higher CA nD reading compared to fresh or refrigerated samples ($P < 0.05$). Whole nD reading from frozen samples were similar to fresh samples, but higher than refrigerated samples ($P < 0.05$).

Nutrient composition and bacterial contamination of MC by breed, lactation and storage method are presented in Table 3. No differences in nutrient content were detected between Holsteins and Jerseys; however, differences were detected within lactation groups and storage methods.

Correlation coefficients for all samples

Correlations between the nD of the CA test supernatant, whole MC and RID were determined using correlation plots, regression equations were then determined to create test dependent equations to determine IgG concentration from nD.

Caprylic Acid Quicktest

The IgG concentration of MC and nD of CA test supernatant (Figure 2) were weakly correlated ($r = 0.53$; $P < 0.0001$, $n = 805$). Impact of lactation number suggests the strongest relationship between CA test nD and RID occured in samples from second lactation cattle (Figure 3). When MC samples were separated by breed (Figure 4), the results suggest that the relationship between CA test nD and actual IgG concentration, may be stronger in Holsteins ($r = 0.61$, $P < 0.0001$) compared to Jersey cattle ($r = 0.49$, $P < 0.0001$). Separating MC samples into groups based on how they were stored (Figure 5) prior to analysis suggests that fresh samples provide the strongest relationship between nD and IgG concentration (r = 0.71, *P* < 0.001) compared to refrigerated (r = 0.56, *P* < 0.0001) and frozen (r = 0.48, *P* < 0.0001) samples.

Whole colostrum

There was a strong relationship (r = 0.73, *P* < 0.0001) between IgG concentration and nD of whole MC (Figure. 6). Maternal colostrum samples from 1st and 2nd lactation cattle (Figure 7) provided similar relationships between nD and actual IgG concentration (r = 0.81, *P* < 0.0001, for both groups respectively). However, samples from 3rd or greater lactation cattle resulted in a weaker relationship between nD and actual IgG concentration (r = 0.69, *P* < 0.0001). Breaking samples into groups based on breed (Figure 8) resulted in similar relationships between nD and actual IgG concentration for samples from Jerseys and Holsteins. Whole MC that were fresh and analyzed for nD had a stronger relationship between nD and IgG concentration (r = 0.71, *P* < 0.0001) compared to refrigerated (r = 0.56, *P* < 0.001) and frozen (r = 0.47, *P* < 0.01) whole MC samples (Figure 9).

Correlation of RID to colostrometer reading

The relationship between actual IgG concentration and the colostrometer estimated IgG concentration was moderate (r = 0.45; n = 78, *P* < 0.0001).

Correlations between RID, nD and nutrients

The RID estimated IgG concentration of MC was moderately correlated with protein (r = 0.69, p < 0.0001, n = 555), TS (r = 0.41, p < 0.001, n = 509), MUN (r = 0.41, p < 0.001, n = 530) and negatively correlated with lactose (r = -0.48, p < 0.0001, n = 551).

The CA test nD reading was highly correlated with the CA test brix reading ($r = 0.99$, $p < 0.0001$, $n = 811$), whole MC nD ($r = 0.60$, $p < 0.0001$, $n = 802$) and moderately correlated with protein ($r = 0.54$, $p < 0.0001$, $n = 544$).

The whole MC nD reading was highly correlated with the whole MC brix reading ($r = 0.99$, $p < 0.0001$, $n = 824$), protein ($r = 0.81$, $p < 0.001$, $n = 546$) and moderately correlated with lactose ($r = -0.53$, $p < 0.001$, $n = 542$) and TS ($r = 0.59$, $p < 0.001$, $n = 500$).

Diagnostic test characteristics for all colostrum samples

The nD of the CA test supernatant and nD of whole MC were analyzed for test characteristics against RID (Table 4). A positive sample was defined as containing an IgG concentration above the cut-point of 50 mg/ml as determined by the RID gold standard test. For assessment of CA test nD and whole MC nD, cut-points were used for nD values that would indicate quality MC (IgG greater than 50 mg/ml). For the CA test, the highest value for sensitivity was found to be at the 1.33987 cut-point (98.57%). The highest value for specificity was found to be at the 1.34242 cut-point (73.28%). For whole MC the cut-point of 1.35966 provided high sensitivity and specificity values (93.58 and 92.24%, respectively).

Diagnostic test characteristics for colostrum samples from Holsteins and Jerseys

Diagnostic test characteristics were assessed for MC from Holstein and Jersey cattle (Table 5). Samples that were not identified by breed or were from crossbred cattle were not used for this analysis. Holsteins and Jerseys followed a similar trend for CA test cut-points. Both breeds had higher sensitivity at the 1.33987 cut-point (98.5 and 96.88%, respectively) and higher specificity at the 1.34242 cut-point (79.02 and 76.19%, respectively).

Additionally the cut-point of whole MC provided high sensitivity (92.96 and 90.63%, respectively) and specificity (90.91 and 100%, respectively) for both breeds.

Diagnostic test characteristics for colostrum samples from 1st, 2nd and 3rd and greater lactations.

Diagnostic test characteristics were also assessed for MC from different lactations (Table 6). Samples that were not identified by lactation, or were pooled were not used in this analysis. Following a similar trend as the overall data set and breed data, the CA test cut-point of 1.33987 provided the greatest sensitivity, and was equal across all lactation groups (96.88%). The 1.34242 cut-point provided the greatest specificity, 68.21% (1st lactation), 82.61% (2nd lactation) and 88.89% (3rd + lactation), respectively. The whole MC cut-point provided high sensitivity (92.96, 90.63 and 90.63%) and high specificity (90.91, 100 and 100%) for 1st, 2nd and 3rd + lactations, respectively.

Diagnostic test characteristics for colostrum samples based on storage

Diagnostic test characteristics of both the CA quick-test and whole MC nD were assessed for based on storage differences (Table 7). Following the same trend as breed and lactation data, the CA test cut-point of 1.33987, provided the greatest sensitivity across fresh, refrigerated and frozen samples (97.48, 99.17 and 98.7%, respectively) and the 1.34242 cut-point provided the greatest specificity across fresh, refrigerated and frozen samples (85.51, 70.73 and 63.69%, respectively). The whole MC cut-point of 1.3566 provided a high sensitivity (93.28, 96.03 and 92.84%) and specificity (88.41, 86.05 and 94.6%) across fresh, refrigerated and frozen samples, respectively.

Accuracy of refractive index as an indicator of IgG concentration

Accuracy of the refractive index of CA test supernatant and whole MC as indicators of IgG status were determined by using the regression equations created from this set of samples (FARMEST and WEST) as well as previous research (LABEST) (Morrill et al., 2011 – unpublished).

The LABEST equation accurately estimated 34.3% of samples within 10 mg/ml of the actual IgG concentration (Table 8). Nutrient LSMEANS for each accuracy group were analyzed (Table 9) for differences. While there were some nutrient differences between groups, no nutrient had a positive or negative impact on the accuracy of the test. The IgG concentration did have an impact on accuracy of the LABEST equation. For samples that were underestimated by the LABEST equation, IgG concentration increased ($P \leq 0.05$) as the accuracy of the LABEST equation decreased. Samples that were overestimated followed a similar trend, with samples estimated within 20 mg/ml having lower ($P \leq 0.05$) IgG concentrations compared to samples with > 20 mg/ml difference from the actual IgG concentration.

The FARMEST equation estimated 30.89% of samples within 10 mg/ml of the actual IgG concentration (Table 10). Nutrient data for FARMEST accuracy groups are presented in Table 11. For underestimated groups, the accuracy difference increased as IgG increased ($P \leq 0.05$). For overestimated groups, the accuracy difference increased as IgG decreased from groups 2 to 4 ($P < 0.005$), and 4 to 6 ($P < 0.005$). No difference in IgG was observed between groups 6 and 8. Protein increased as underestimated groups became more inaccurate. Protein is lowest ($P \leq 0.05$) in samples that are overestimated by > 30 mg/ml. Lactose concentration increased ($P \leq 0.05$) as accuracy decreased from within 10 mg/ml to greater than 20 mg/ml and greater than 30 mg/ml of the actual IgG concentration. Samples that are overestimated by greater than 30 mg/ml have the lowest amount of TS content compared to all other groups ($P < 0.005$). Other solids content decreases as accuracy decreased from within 20 mg/ml to greater than 20 mg/ml of the actual IgG concentration. No differences were observed in TPC between underestimated groups. The SCClog for underestimated samples decreases, as accuracy decreases, while it increases for overestimated samples.

The WEST equation for whole MC had the greatest accuracy of the 3 equations, with 43.77% of samples estimated within 10 mg/ml of the actual IgG concentration and over 70% estimated within 20 mg/ml of the actual IgG concentration (Table 12). Nutrient means and differences of WEST accuracy groups are presented in Table13. The IgG concentration increased ($P \leq 0.05$) as samples were underestimated within 10 mg/ml, to within 20 mg/ml to greater than 20 or 30 mg/ml, while IgG decreased ($P \leq 0.05$) as overestimation increased. Protein increased slightly as underestimation increased. Lactose increased slightly as overestimation increased. No differences were observed for TPC or SCC.

Accuracy groups for all 3 equations were evaluated for breed (Table 14) lactation (Table 15) and storage (Table 16) differences. Across all test equations, Jerseys had a greater percentage ($P < 0.001$) of samples underestimated for IgG concentration in MC as compared to Holsteins. A greater percentage ($P < 0.05$) of Holstein MC samples were classified within 10 mg/ml of the actual IgG concentration as compared to Jersey samples using the LABEST equation. The LABEST and WEST equations resulted in similar percentages of Holstein and Jersey samples within each accuracy group.

DISCUSSION

Samples of MC collected for this study represent what was available at that particular time and may not be representative of fresh MC produced by dairy cattle in the U.S. Samples were collected only from dairies that did not pasteurize or add preservative to MC; additionally managers/herd owners had to be willing to complete a management survey. This is believed to be the first study in which the nD values are reported and not brix values. Brix values are determined by applying the nD to a regression equation that corresponds to the sugar, alcohol or protein content of a solution.

Correlations:

Providing a tool for producers to rapidly and accurately estimate IgG status of MC could potentially improve the health of calves and profitability of producers. Earlier work from our lab reported that the CA test provided a strong correlation ($r = 0.96$, $P < 0.0001$, $n = 30$) between nD of CA test supernatant and RID obtained IgG concentration in the laboratory (Morrill et al., unpublished). This relationship was not as strong within the on-farm data set. The strongest relationship between CA supernatant nD and actual IgG concentration was observed in fresh MC samples. Overall the nD of whole MC from 1st and 2nd lactation cattle provided the strongest correlation. A refractometer can provide a reasonable estimation ($R^2 = 0.76$) of IgG concentration when whole MC is 20^0C (Mechor et al., 1992). Research from the University of Guelph supported these resulted by reporting that optical and digital Brix refractometers were highly correlated for both fresh and frozen samples ($r = 0.98$; $n = 288$) with a correlation coefficient between 0.71 and 0.74 when compared to IgG concentration determined by RID (Bielmann, 2010). Additional research has reported slightly lower correlation coefficients (r = 0.62, n = 171) of IgG determined by RID and refractometry (Chigerwe et al., 2008). Both of these datasets are based off of MC samples collected from Holstein cattle. No literature was available on the relationship between nD and IgG concentration in Jersey or crossbred cattle.

Correlating mare colostrum (n = 66) %Brix readings on a refractometer with IgG concentrations determined by immunotubidometric methods resulted in a strong correlation (r = 0.94; Cash, 1999). The high correlation coefficient may potentially be due to the small sample number; additionally, samples were centrifuged prior to analysis to separate the whey from the fat portion. Analysis (refractometry and immunotubidometric methods) was then completed on only the whey fraction, thus eliminating the confounding factor of nutrient composition. Removing casein and fat during whey preparation of bovine MC causes a concentrating effect of IgG, resulting in an exaggerated precipitin ring during RID analysis (Fleenor and Stott, 1981).

The data from the current study suggest that breed and lactation have a minimal impact on the relationship between CA test supernatant nD and actual IgG concentration and that storage of the MC prior to analysis significantly affects the correlation. For analysis of whole MC, the relationships reported in this data set may be weaker than previously reported (Mechor et al, 1992). However, all relationships excluding 3rd and greater lactation, refrigerated and frozen MC samples provided similar (Bielmann, 2010) or greater (Chigerwe et al, 2008) correlation coefficients then previously reported. This data suggests that breed does not impact the relationship between nD and actual IgG, where as storage method and potentially lactation does impact the relationship.

Diagnostic test characteristics

To quickly determine if MC is of high enough quality to feed to calves, nD cut-point levels need to be determined that strongly correspond to RID values greater than 50 mg/ml. Diagnostic test characteristics were established for CA quick-test nD and whole MC nD based off of the entire data set, by breed, lactation and storage method to determine if there should be different cut-points for different variants. Maximizing the sensitivity of a test allows for the smallest number of samples being inaccurately identified as greater than 50 mg/ml. This prevents the feeding or storage of MC with insufficient IgG (less than 50 mg/ml) thus making sensitivity of a MC quality test more important than specificity. Use of cut-point values lower than optimal would increase the amount of MC classified as adequate, but would increase the chances that inadequate MC would be classified as adequate. Using cut-points greater then optimal, would decreases the chances of inaccurately classifying poor MC as adequate, however some adequate MC may be incorrectly classified as inadequate.

Utilizing brix values, a cut-point of 22% for whole MC was determined to be optimal and resulted in a sensitivity of 75% and specificity of 78% (Chigerwe et al., 2008). Additional research has confirmed that the 22% cutpoint provides optimal sensitivity (90.5%) and specificity (85.0%) when a digital refractometer is used (Bielmann et al., 2010). The sensitivity (93.58%) and specificity (92.24%) for whole MC in this data set (n = 827), using a cut-point of 1.35966 nD, were greater than previously reported brix data. This suggests that the 1.35966 cut-point is highly reliable to identify quality MC. Additionally the recommended, digital brix cut-point of 22% results in a high sensitivity (90.1%) and high sensitivity and specificity (92.2 and 83.3%, respectively) for first lactation heifers and cows

(Bielmann et al., 2010). No differences in diagnostic test characteristics were observed across breed for the CA test cut-point of 1.33987 or the whole MC cut-point, there was a trend for MC samples from Holsteins to have a higher sensitivity and NPV compared to Jersey samples when the CA test cutpoint of 1.34242 was utilized. The cut-point of 1.35966 nD provided high sensitivity and specificity across breed, lactations and storage methods. This suggests that parity, breed and storage do not have an impact on the accuracy of nD on determining if whole MC is adequate.

The CA test cut-point, 1.34242, resulted in sensitivity and specificity results similar (77.18 and 73.28%, respectively) to that reported by Chigerwe (2008) in whole MC. The lower cutpoint of 1.3398 for the CA test, resulted in a sensitivity of 98.57%, but the lower specificity suggests that a large number of samples are being classified as inadequate and have actual IgG concentration greater than 50 mg/ml. The cut-point of 1.33987 nD resulted in high sensitivities across lactations, breeds and storage methods. This suggests that parity, breed and storage do not have an impact on the accuracy of the CA test on determining if MC is adequate.

Accuracy in calculating IgG from nD

Alcohol and sugar refractometers have been adapted for use in the dairy industry by creating cut-points that estimate if the MC is of adequate quality, unfortunately actual IgG concentration is still unknown to the producer. It would be of great value to dairy producers to have a refractometer that directly measured the IgG concentration of MC, and did not just provide an nD or brix reading that was used to determine if the MC was of adequate quality. Using the LABEST equation, the accuracy of determining the IgG concentration of MC based off of the nD, allowed for 34.3% of samples to be estimated within 10 mg/ml of the RID determine IgG concentration, however 24.21% of samples were underestimated by greater than 20 mg/ml and 17.78% of samples were overestimated by greater than 20 mg/ml of the RID determined IgG concentration. The FARMEST equation resulted in 19.05% of sample to be underestimated and 22.06% to be overestimated by greater than 20 mg/ml. The WEST equation had the greatest accuracy with 71.77% of samples being estimated within 20 mg/ml of the RID obtained IgG concentration. As IgG concentration moved away from the mean, the accuracy of all three equations decreased, with underestimated samples increasing in IgG and overestimated samples decreasing in IgG. This suggests that IgG concentration may impact the accuracy of the CA test and nD of whole MC.

Fat concentration may impact the Brix reading (Fox and Sweeney 1998; Dinsmore and Skidmore, 2008). No differences in fat means were detected across accuracy groups when the LABEST equation was used to estimate IgG concentration. Extremely high fat content was observed in samples that were overestimated 20 to 30 mg/ml from the actual IgG concentration. Whole MC nD accuracy groups did have different fat concentrations, with greater fat concentration observed in overestimated samples. This suggests that fat has a minimal impact on the CA test accuracy, and may impact the nD of whole MC.

Due to the small number of samples collected from crossbred dairy cattle it is not possible to determine the accuracy of the CA test or whole MC nD for estimating IgG concentration. A greater percentage of MC samples from Holsteins were estimated within 10 mg/ml and there was a trend for more Holsteins to be estimated within 10 to 20 mg/ml using the LABEST equation as compared to Jersey samples. A greater percentage of MC samples from Jerseys were inaccurately estimated by greater than 30 mg/ml of the actual IgG compared to Holsteins. Additionally a greater percentage of Jersey samples were underestimated as compared to MC samples from Holsteins. The FARMEST and WEST equations resulted in similar percentages of samples being estimated across all four groups for Holsteins and Jerseys, however a greater percentage of MC samples were underestimated by greater than 30 mg/ml, and a greater percentage of total MC samples from Jerseys were underestimated compared to MC samples from Holsteins.

Lactation

Analysis of whole MC had the greatest correlation for fresh samples followed by refrigerated and then frozen samples, conversely analysis by the CA test had the greatest accuracy for fresh, followed by frozen and then refrigerated samples. Fresh samples had greater lactose, OS, SCC, and TPC and lower protein content compared to the other storage groups. The coliform content of MC that was refrigerated prior to feeding had the greatest TPC and coliform count as compared to the other groups. This suggests that bacteria may interfere with the CA precipitation of IgG, but does not impact the nD of whole MC.

Throughout this study is was assumed that the RID analysis for IgG is accurate for determining IgG concentration in MC. Fleenor and Stott (1981) commented that RID analysis has not been investigated thoroughly to determine sources of potential error. While RID continues to be the industry gold standard to determine IgG concentration, limited information is available on what can potentially lead to inaccuracies and it is not possible to determine if factors that affected the relationship between RID-determined IgG and refractometry estimated IgG were affecting the accuracy of the RID kits or the refractometer.

CONCLUSION

The objectives of this study were to validate the lab results of the CA test with an on farm trial, determine the impacted of breed, parity, storage method, nutrient composition and bacterial contamination and compare the CA test to refractometry of whole MC. This study concludes that breed, lactation and nutrient composition do not impact the accuracy of the CA test or nD of whole colostrum to estimate IgG concentration in MC. The storage of MC does impact the accuracy of both tests, and samples should be analyzed fresh to get the most accurate results. Comparing the two testing methods suggests that the nD of whole MC provides the most accurate and rapid estimate of IgG concentration compared to the CA test.

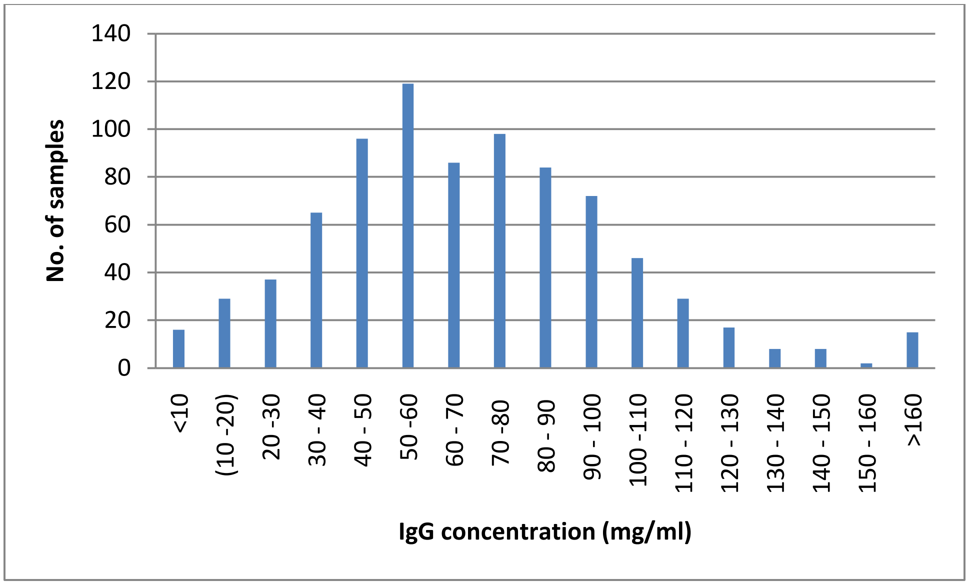


Figure 1. A distribution plot of the radial immunodiffusion assay determined IgG concentration of 827 colostrum samples.

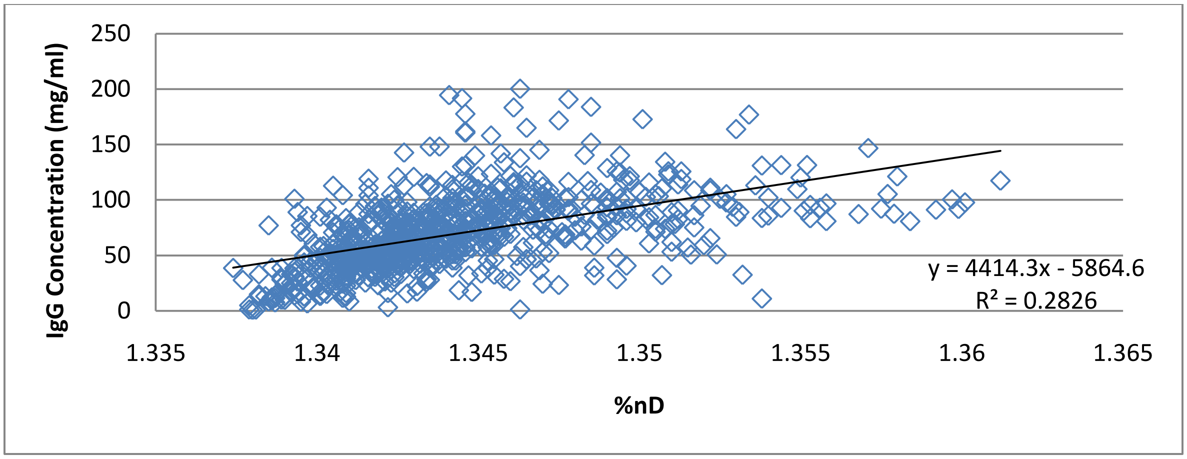


Figure 2. A regression plot between the quick-test %nD and the laboratory RID analysis for IgG from colostrum (n = 805).

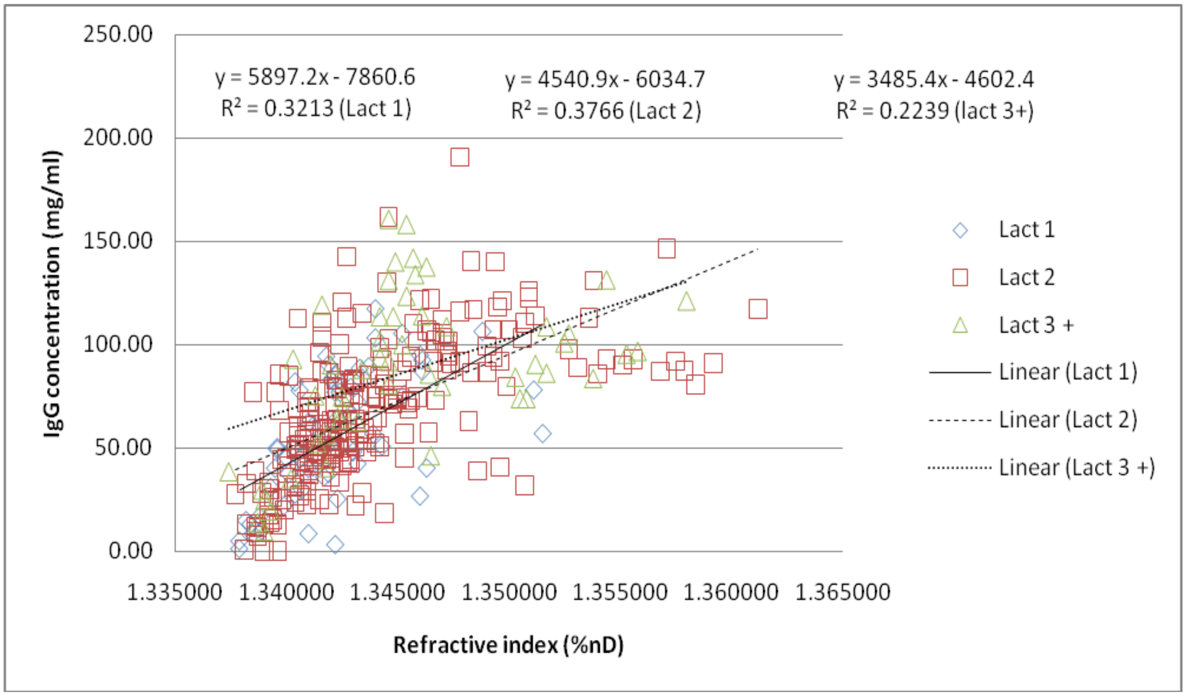


Figure 3. A regression plot between the quick-test %nD and the laboratory RID analysis of IgG from colostrum across lactations (n = 351; lactation 1 = 49, lactation 2 = 174, lactation 3 = 128).

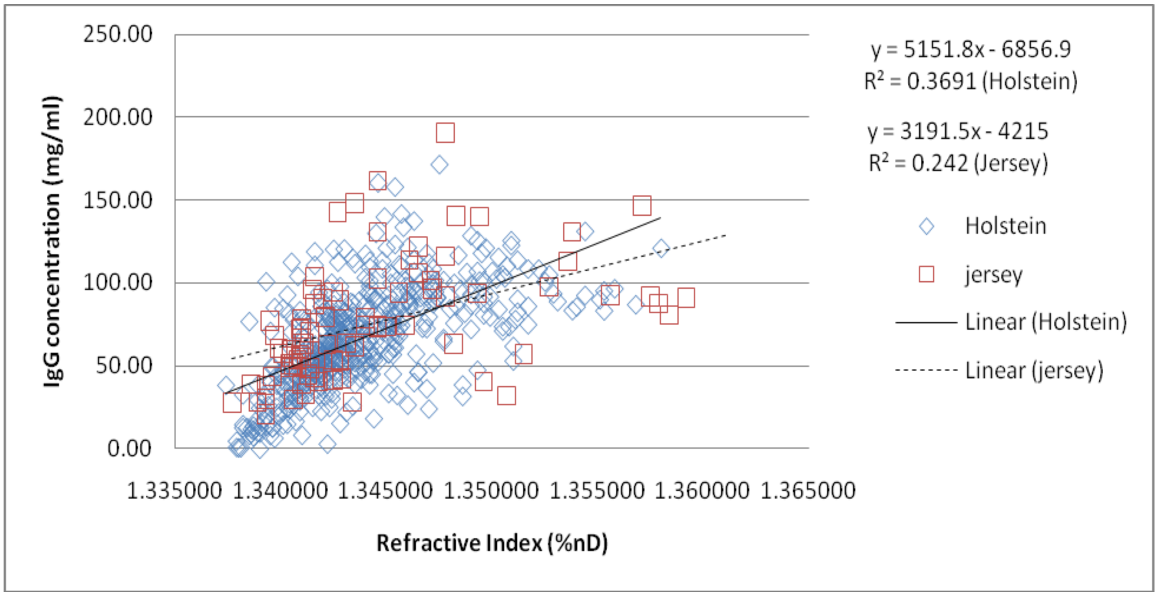


Figure 4. A regression plot between the quick-test %nD and the laboratory RID analysis of IgG from colostrum across breeds (n = 570; Holstein = 485, Jersey = 85).

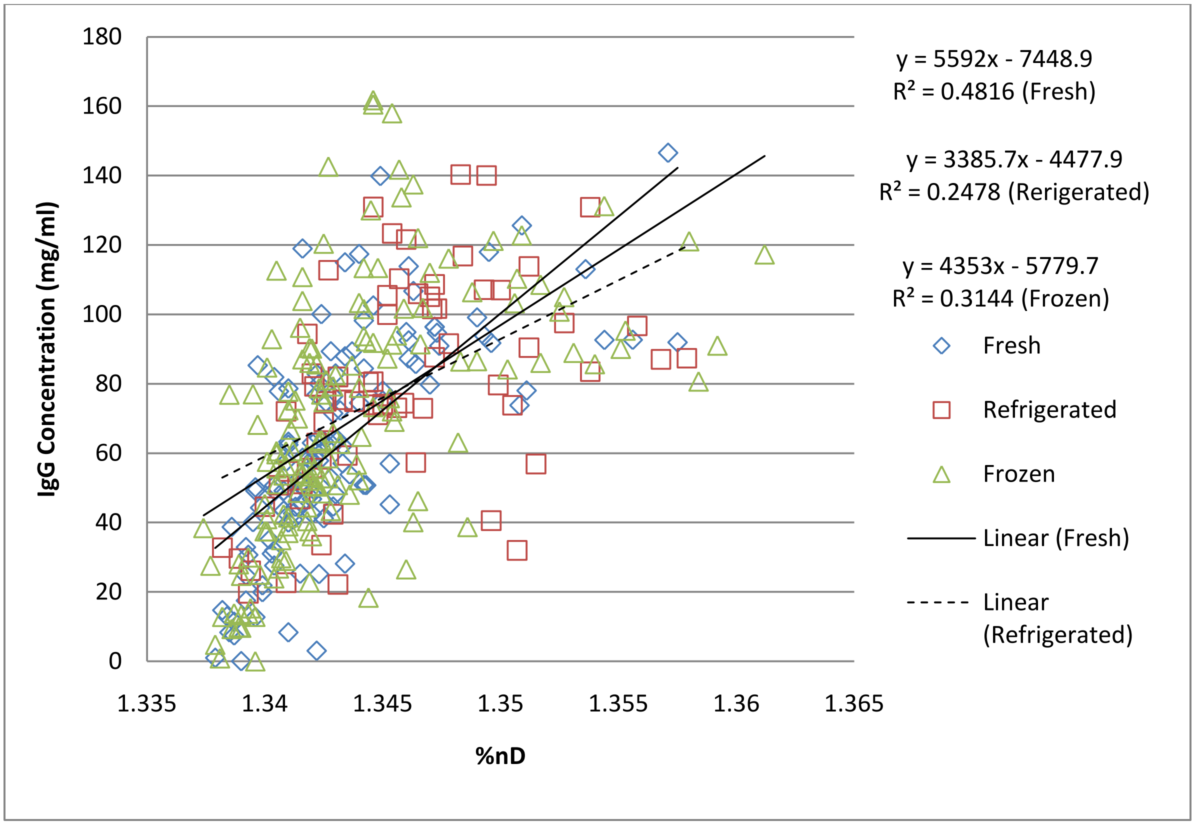


Figure 5. A regression plot between the quick-test %nD and the laboratory RID analysis of colostrum across methods of storage (n = 827; fresh = 196, refrigerated = 152, frozen = 479).

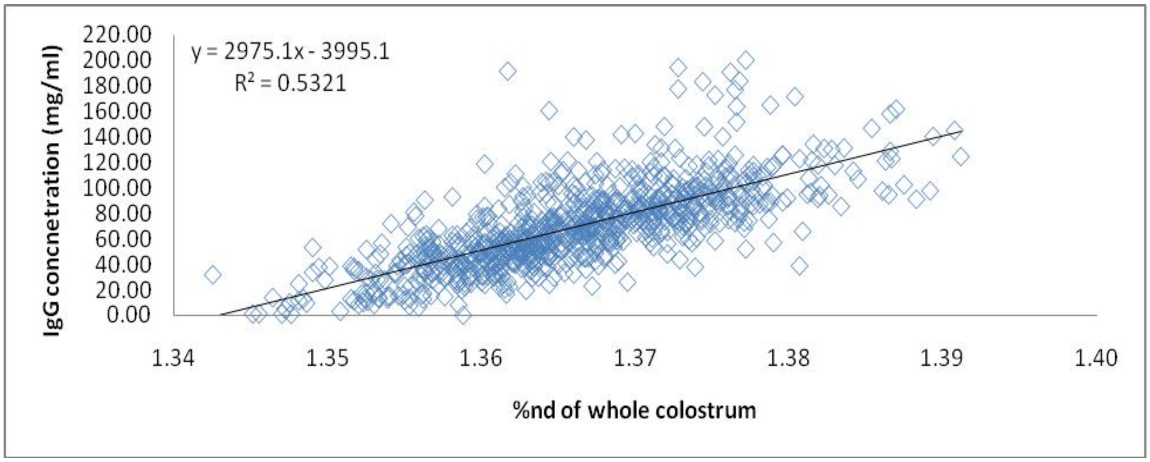
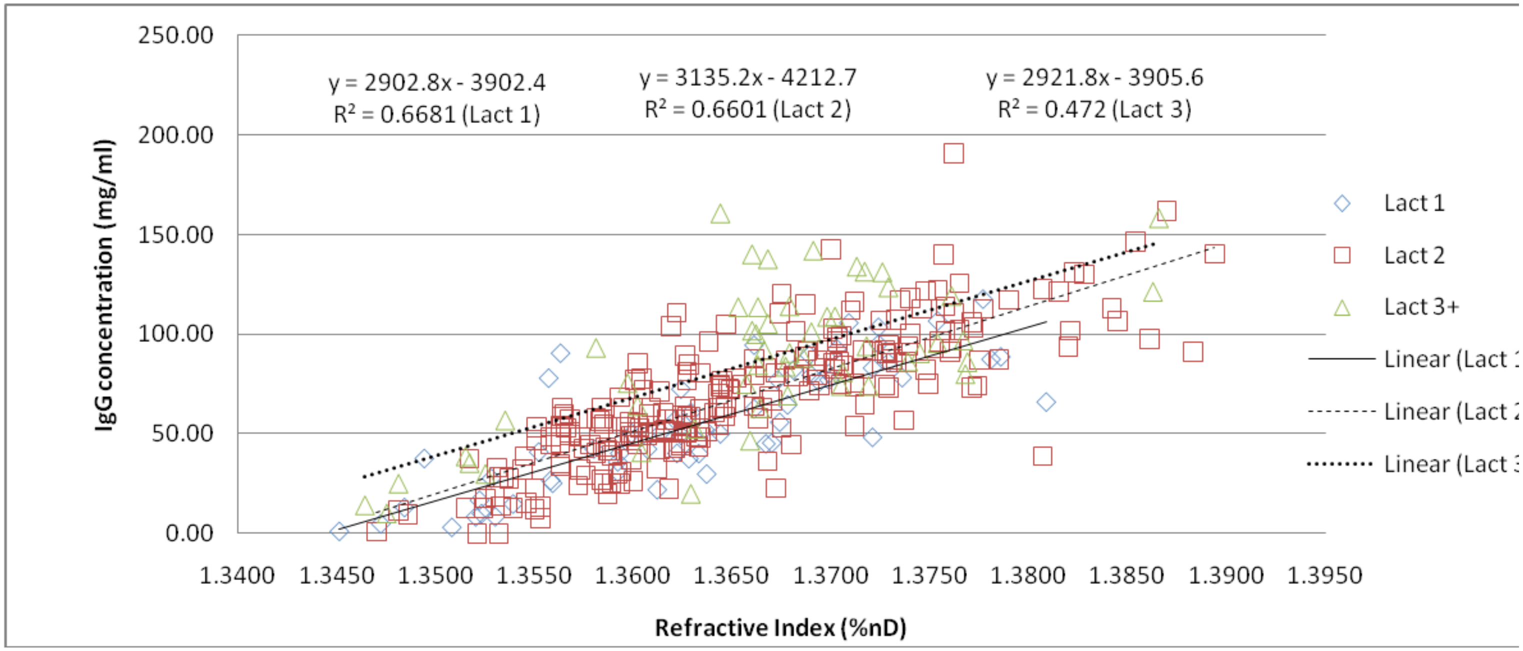


Figure 6. Whole colostrum nD reading compared to IgG concentration determined by RID assay. (n = 823).

Figure 7. A regression plot between %nD of whole colostrum and the laboratory RID analysis of IgG from colostrum across lactations (n = 351; lactation 1 = 49, lactation 2 = 174, lactation 3 = 128).



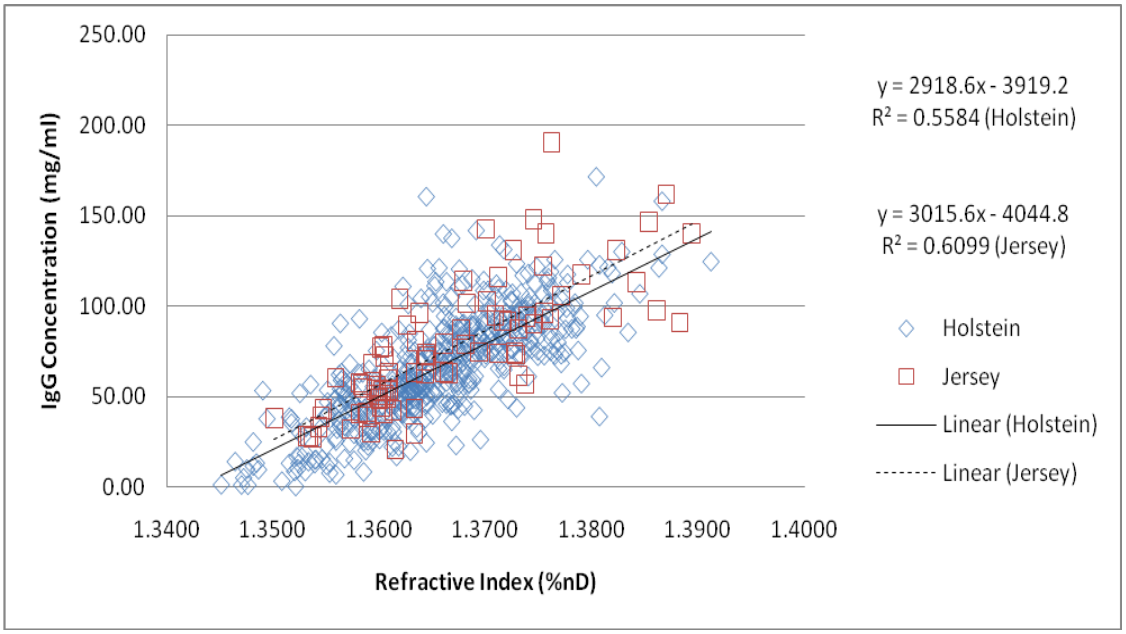


Figure 8. A regression plot between %nD of whole colostrum and the laboratory RID analysis of IgG from colostrum across breeds (n = 570; Holstein = 485, Jersey = 85).

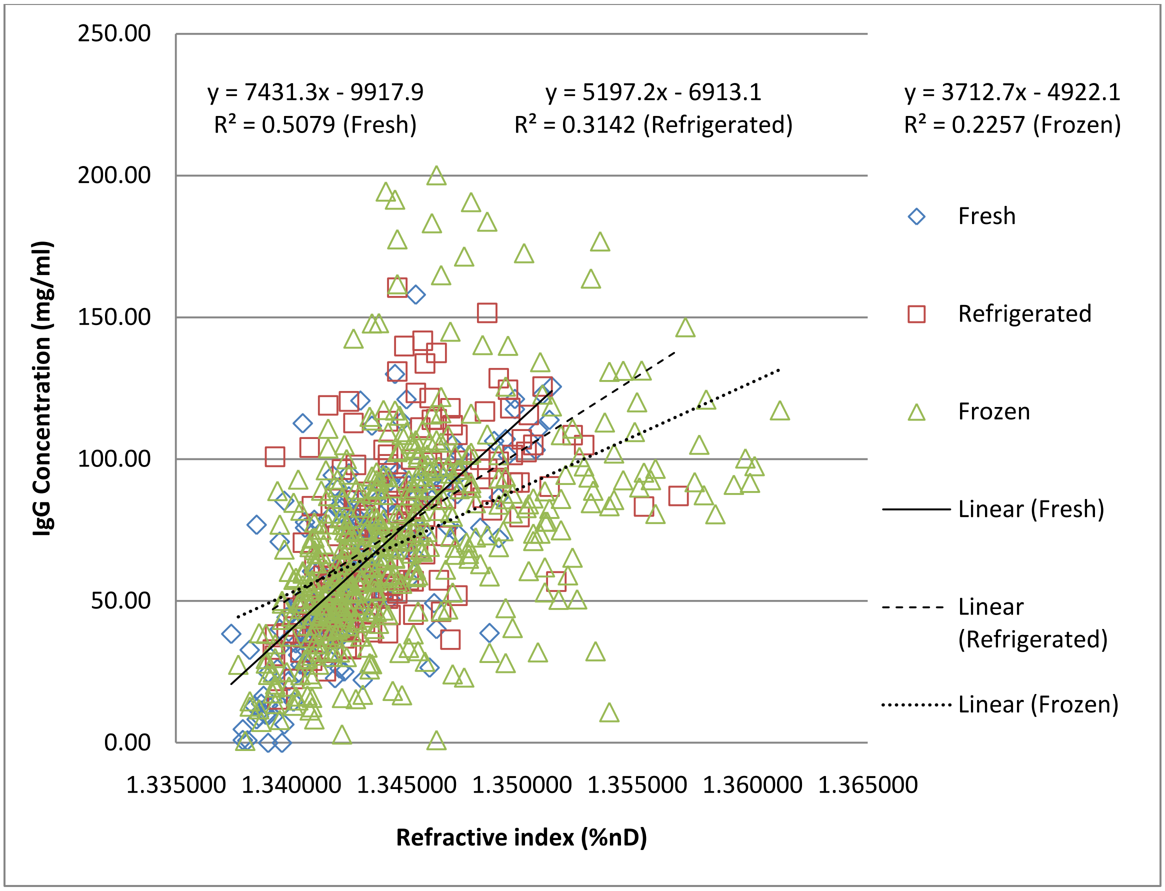


Figure 9. A regression plot between %nD of whole colostrum and the laboratory RID analysis of IgG from colostrum across methods of storage (n = 827; fresh = 196, refrigerated = 152, frozen = 479).

Table 1. Colostrum samples collected across state, breed, lactation number, milking number post calving and storage method prior to feeding

			Breed				Lactation					Milking post calving			Storage method[1]		
State	farms	samples	holstein	Jersey	crossbred	n/a	1	2	3	4+	n/a	1	2	3	1	2	3
AZ	2	61	40	~	~	21	2	7	~	~	52	61	~	~	7	9	45
CA	14	173	161	8	~	4	34	76	~	~	63	169	4	~	93	34	46
FL	4	35	33	2	~	~	1	22	~	~	12	30	~	~	17	9	9
GA	2	30	30	~	~	~	~	30	~	~	~	30	~	~	11	19	~
IA	1	40	40	~	~	~	~		~	~	40	40	~	~	~	~	40
MN	11	97	35	~	~	62	8	10	4	5	70	97	~	~	2	6	89
NH	1	18	18	~	~	~	~	6	3	9	~	6	6	6	18	~	~
NY	5	59	41	3	~	15	4	11	4	2	38	49	5	5	17	29	13
PA	5	51	42	9	~	~	~	5	17	22	7	46	4	1	~	23	28
TX	9	176	5	62	7	102	~		61	~	115	175	1	~	6	3	167
VA	7	60	22	3		35	~	2	1	~	57	60	~	~	25	20	15
WI	6	27	27	~	~	~	~	5	~	~	22	26	1	~	~	~	27
TOTAL	**67**	**827**	**494**	**87**	**7**	**239**	**49**	**174**	**90**	**38**	**476**	**789**	**21**	**12**	**196**	**152**	**479**

[1] How the sample was stored prior to analysis by the caprylic acid test. 1 = fresh (not stored), 2 = refrigerated, 3 = frozen

TABLE 2. Measurements and estimates of IgG concentration in samples by breed, lactation and storage method

	Breed			Lactation				Storage method			
	Holstein	Jersey	SE	1	2	3+	SE	fresh	refrigerated	frozen	SE
IgG (mg/ml)[1]	74.16	65.77	8.33	42.39^{a}	68.57^{b}	95.87^{c}	9.3	69.04	74.55	66.31	7.34
Quicktest %nD	1.34424	1.34646	0.0013	1.34608	1.34525	1.34472	0.0014	1.34337^{a}	1.3435^{a}	1.34916^{b}	0.0011
Whole %nD	1.36537	1.36641	0.0012	1.36618	1.36496	1.366518	0.0015	1.36595^{ab}	1.3648^{a}	1.3669^{b}	0.0096

Differences between means are indicated by different alphabetical superscripts ($P < 0.05$)

[1]As determined by radial immunodiffusion

TABLE 3. Nutrient and bacterial means by breed, lactation and storage method

	Breed			Lactation				Storage method			
	Holstein	Jersey	SE	1	2	3	SE	fresh	refrigerated	frozen	SE
Fat (%)	5.33	5.25	0.50	6.55^{a}	4.2^{c}	5.14^{b}	0.53	4.88	5.37	5.64	0.47
Protein (%)	12.47	12.59	0.67	12.35	12.09	13.14	0.73	10.92^{c}	14.1^{a}	12.55^{b}	0.64
Lactose (%)	2.97	2.93	0.10	2.99^{ab}	2.78^{b}	3.08^{a}	0.10	3.18^{a}	2.75^{b}	2.92^{b}	0.09
Other Solids (%)	4.44	4.40	0.08	4.43^{a}	4.24^{b}	4.59^{a}	0.08	4.56^{a}	4.31^{b}	4.38^{b}	0.07
Total Solids (%)	22.15	22.98	0.90	23.46^{a}	20.83^{b}	23.40^{a}	0.99	21.21^{b}	24.16^{a}	22.33^{b}	0.87
Mun	29.51	27.51	2.13	28.69^{ab}	25.29^{b}	31.55^{a}	2.40	22.99^{c}	34.02^{a}	28.53^{b}	2.06
SCC (*1,000)	2816.72^{a}	1256.24^{b}	510.00	3875.52^{a}	1408.24^{b}	825.68^{b}	587.03	2745.51^{a}	1299.26^{b}	2064.68^{a}	502.57
SCCLog	5.89^{a}	5.33^{b}	0.13	5.99^{a}	5.59^{b}	5.26^{c}	0.15	5.79^{a}	5.46^{b}	5.58^{a}	0.13
Coliform Log	1.53^{a}	1.16^{b}	0.14	1.24^{b}	1.54^{a}	1.26^{b}	0.13	1.12^{b}	1.58^{a}	1.34^{a}	0.13
TPCLog	4.88^{a}	4.11^{b}	0.14	4.49^{ab}	4.70^{a}	4.31^{a}	0.14	3.97^{c}	4.99^{a}	4.54^{b}	0.13

^{abc}Difference between means of each group are indicated by different alphabetical superscripts ($P < 0.05$)

Table 4. Diagnostic test characteristics for the digital refractometer measuring supernatant from the CA test or whole colostrum compared with IgG determined by radial immunodiffusion assay.

Item	nD cut-point	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CA test	1.34242	77.18	73.28	87.47	57.05
CA test	1.33987	98.57	21.98	75.34	86.44
Whole Colostrum	1.35966	93.58	92.24	96.69	85.6

Table 5. Diagnostic test characteristics for the digital refractometer measuring supernatant from the CA test or whole colostrum compared with IgG determined by radial immunodiffusion assay across breeds

		Holstein (n = 485)				Jersey (n = 85)			
Item	cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CA test	1.34242	74.55	79.02	89.25	57.07	64.06	76.19	89.13	41.03
	1.33987	98.5	27.97	76.16	88.89	96.88	33.33	81.58	77.78
Whole colostrum	1.35966	92.96	90.91	95.97	84.41	90.63	100	100	77.78

Table 6. Diagnostic test characteristics for the digital refractometer measuring supernatant from the CA test or whole colostrum compared with the IgG determined by radial immunodiffusion assay across lactations.

		Lact 1 (n = 49)				Lact 2 (n = 172)				Lact 3 (n = 128)			
Item	cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CA test	1.34242	53.12	86.21	80.95	62.5	66.47	82.61	90.24	50.44	83.33	88.89	97.22	53.3
CA test	1.33987	96.88	34.45	62	88.89	96.88	34.78	81.58	77.78	96.88	66.66	81.58	77.78
Whole colostrum	1.35966	92.96	90.91	96.06	84.41	90.63	100	100	77.78	90.63	100	100	77.78

Table 7. Diagnostic test characteristics for the digital refractometer measuring supernatant from the CA test or whole colostrum compared with the IgG determined by radial immunodiffusion assay across methods of storage.

		Fresh (n = 196)				Refrigerated (n = 152)				Frozen (n = 479)			
Item	cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CA test	1.34242	71.43	85.51	89.47	63.44	80.83	70.73	89	55.77	78.18	63.96	85.71	51.45
CA test	1.33987	97.48	34.78	72.05	88.89	99.17	12.2	16.77	83.33	98.7	12.61	75.75	77.78
Whole colostrum	1.35966	93.28	88.41	93.28	88.41	96.03	86.05	95.28	88.1	92.84	94.6	97.94	82.68

Table 8. Percentage of samples over/underestimated using the CA test supernatant refractive index and LABEST equation as an indicator of IgG concentration

	Total % of samples		
	underestimated	overestimated	total
Actual IgG - LABest[1]			
< 10 mg/ml difference	18.79	15.51	34.3
10 - 20 mg/ml difference	14.5	9.21	23.71
20 - 30 mg/ml difference	10.21	4.29	14.5
> 30 mg/ml difference	14	13.49	27.49
TOTAL (%)	57.5	42.5	100

Percentage of samples over and underestimated by the LABEST equation (n = 787).

[1] LABEST = 9576.7 * %nd -12806

Table 9. Nutrient LSMeans for CA test LABEST accuracy groups

	X	Within 10 mg/ml		Within 10 - 20 mg/ml		Within 20 - 30 mg/ml		> 30 mg/ml difference	
		under Group 1	over group 2	under group 3	over group 4	under group 5	over group 6	under group 7	over group 8
n	827	149	123	115	73	81	34	111	141
RID (mg/ml)	68.84	61.22^{b}	51.68^{ab}	69.47^{c}	49.93^{a}	79.02^{d}	59.83^{b}	108.44^{e}	66.32^{bc}
Fat (%)	5.61	5.86	5.73	5.26	6.64	5.41	5.54	5.29	5.34
Protein (%)	12.72^{a}	12.57^{b}	11.49^{a}	12.95^{a}	12.33^{a}	13.21^{a}	12.16^{a}	13.98^{c}	12.86^{a}
Total solids (%)	22.56^{a}	22.28^{a}	21.48^{ab}	22.6^{a}	25.53^{a}	22.66^{a}	22.15^{a}	23.39^{ab}	22.51^{a}
Other solids (%)	4.34	4.36^{a}	4.51^{b}	4.34^{a}	4.29^{a}	4.25^{a}	4.28^{a}	4.16^{c}	4.39^{ab}
Lactose		2.83	3.09	2.84	2.83	2.76	2.79	0.7	2.89
TPClog	4.96	5.02^{a}	4.95^{ab}	5.08^{a}	5.12^{a}	4.71^{b}	4.97^{ab}	4.89^{ab}	4.94^{ab}
SCC log	5.86	5.77^{a}	5.97^{ab}	5.79^{a}	6.02^{b}	5.81^{a}	6.28^{b}	5.58^{ac}	5.91^{a}
Coliform (cfu/ml)	120.86	170.8^{a}	126.72^{a}	129.45^{a}	43.83^{b}	94.29^{b}	25.94^{b}	113.61^{a}	146.63^{a}

$^{a\text{-}e}$Means within a row with different superscripts differ (*P* < 0.05)

TABLE 10. Percentage of samples over/underestimated using the CA test supernatant refractive index and FARMEST equation as an indicator of IgG concentration

	Samples (%)[1]		
	underestimated	overestimated	total
Actual IgG - FARMEST IgG[2]			
< 10 mg/ml difference	12.48	18.41	30.89
10 - 20 mg/ml difference	12.36	15.64	28
20 - 30 mg/ml difference	8.08	10.59	18.67
> 30 mg/ml difference	10.97	11.47	22.44
TOTAL (%)	43.89	56.11	100

[1] n = 787

[2] ONFARMEST = 4414.3 * %nd - 5864.6

Table 11. Nutrient LSMeans for CA test FARMEST accuracy groups

		Within 10 mg/ml		Within 10 - 20 mg/ml		Within 20 - 30 mg/ml		> 30 mg/ml difference	
	Overall means	under Group 1	over group 2	under group 3	over group 4	under group 5	over group 6	under group 7	Over group 8
n	827	99	146	98	124	64	84	87	125
RID	68.84	74.28^{d}	61.02^{c}	85.89^{e}	51.72^{b}	96.18^{f}	44.79^{a}	124.58^{g}	40.67^{a}
Fat	5.61	5.79	5.38	5.02	5.67	5.72	6.93^{a}	4.92	5.45
Protein	12.72	14.08^{c}	12.38^{b}	13.37^{c}	11.5^{b}	14.75^{cd}	11.85^{b}	15.31^{d}	10.2^{a}
Lactose		2.72^{ab}	2.85^{bc}	2.75^{b}	2.94^{c}	2.59^{ab}	3.09^{d}	2.58^{a}	3.13^{d}
Total solids	22.56	24.13^{d}	22.17^{bc}	23.24^{cd}	21.29^{b}	24.89^{d}	23.29^{cd}	23.54^{cd}	19.84^{a}
Other solids	4.34	4.29^{bc}	4.37^{bc}	4.34^{bc}	4.36^{bc}	4.15^{ab}	4.53^{d}	4.03^{a}	4.46^{cd}
TPC log	4.96	5.01^{ab}	4.97^{ab}	4.79^{ab}	5.13^{bc}	5.04^{ab}	5.08^{bc}	4.92^{ab}	4.79^{ab}
SCC log	5.86	5.87^{bc}	5.87^{bc}	5.77^{b}	5.98^{cd}	5.64^{ab}	6.01^{cd}	5.44^{a}	6.12^{d}
Coliform	120.86	111.59^{ab}	117.43^{ab}	52.92^{a}	114.87^{ab}	39.89^{a}	158.53^{b}	144.7^{ab}	191.13^{b}

$^{a\text{-}e}$Means within a row with different superscripts differ ($P < 0.05$)

Table 12. Percentage of samples over/underestimated using whole colostrum refractive index as an indicator of IgG concentration

	total % of samples[1]		
	underestimated	overestimated	total
Actual IgG - West IgG[2]			
< 10 mg/ml difference	19.73	24.04	43.77
10 - 20 mg/ml difference	10.98	17.02	28
20 - 30 mg/ml difference	5.18	9.25	14.43
> 30 mg/ml difference	8.38	5.42	13.8
TOTAL (%)	44.27	55.73	100

[1] n = 787

[2] West = 0.2975.1* %nd - 3995.1

Table 13. Nutrient LSMeans for WEST accuracy groups

		Within 10 mg/ml		Within 10 - 20 mg/ml		Within 20 - 30 mg/ml		> 30 mg/ml difference	
		under Group 1	over group 2	under group 3	over group 4	under group 5	Over group 6	under group 7	Over group 8
n	827	160	195	89	138	42	75	68	60
RID	68.84	73.89^{d}	61.44^{c}	81.34^{e}	51.96^{b}	96.97^{f}	43.04^{a}	122.42^{g}	51.57^{b}
Fat	5.61	5.22^{ab}	5.64^{bc}	4.68^{a}	6.5^{c}	4.83^{ab}	7.49^{c}	4.82^{ab}	5.47^{abc}
Protein	12.72	12.98^{b}	12.11^{a}	12.42^{ab}	12.36^{ab}	13.61^{bc}	11.73^{a}	14.48^{c}	13.43^{bc}
Lactose		2.73^{ab}	2.95^{cd}	2.86^{bc}	2.89^{cd}	2.75^{abc}	3.09^{d}	2.68^{a}	2.89^{bcd}
Total solids	22.56	22.09^{ab}	22.3^{abc}	21.53^{a}	22.94^{abc}	22.64^{abc}	23.12^{abc}	23.72^{c}	23.69^{bc}
Other solids	4.34	4.19^{a}	4.44^{c}	4.34^{abc}	4.35^{bc}	4.30^{abc}	4.48^{c}	4.23^{ab}	4.42^{bc}
TPClog	4.96	5.03	4.93	4.91	5.04	4.95	4.98	4.88	4.88
SCC log	5.86	5.78	5.89	5.87	5.98	5.65	5.92	5.7	5.98
Coliform	120.86	82.67^{a}	127.61^{ab}	103.78^{ab}	139.36^{ab}	95.3^{ab}	183.74^{b}	102.85^{ab}	127.43^{ab}

$^{a-e}$Means within a row with different superscripts differ ($P < 0.05$)

Table 14. Percentage of maternal colostrum samples over/underestimated by LABEST, FARMEST and WEST across Holstein and Jersey samples.

	Holstein			Jersey			P-Value		
	total % of samples			total % of samples					
	under - estimated	over - estimated	total	under - estimated	over - estimated	total	under - estimated	over - estimated	Total
Actual IgG - LABest[1]									
< 10 mg/ml difference	20.24	17.00	37.24	16.47	9.41	25.88	0.464	0.079	0.049
10 - 20 mg/ml difference	15.00	9.11	24.11	14.12	1.18	15.30	1.000	0.008	0.092
20 - 30 mg/ml difference	9.51	3.64	13.15	15.29	2.35	17.64	0.122	1.753	0.306
> 30 mg/ml difference	11.94	13.56	25.50	24.71	16.47	41.18	0.003	0.498	0.006
TOTAL (%)	56.69	43.31	100	70.59	29.41	100	0.0171	1.017	
Actual IgG - FARMEST									
< 10 mg/ml difference	13.16	19.03	32.19	16.47	17.65	34.12	0.396	0.880	0.708
10 - 20 mg/ml difference	11.94	15.38	27.32	12.94	9.41	22.35	0.857	0.182	0.425
20 - 30 mg/ml difference	7.29	9.92	17.21	7.06	9.41	16.47	1.000	1.000	1.000
> 30 mg/ml difference	9.11	14.17	23.28	17.65	9.41	27.06	0.032	0.303	0.491
TOTAL (%)	41.5	58.5	100	54.12	45.88	100	0.033	0.033	
Actual IgG - WEST[3]									
< 10 mg/ml difference	19.23	24.50	43.73	23.53	24.71	48.24	0.378	1.000	0.479
10 - 20 mg/ml difference	11.94	17.61	29.55	11.76	10.59	22.35	1.000	0.117	0.195
20 - 30 mg/ml difference	5.26	9.11	14.37	8.24	2.35	10.59	0.307	0.032	0.339
> 30 mg/ml difference	6.07	6.28	12.35	12.94	5.88	18.82	0.036	1.000	0.119
TOTAL (%)	42.5	57.5	100	56.47	43.53	100	0.018	0.018	

Table 15. Percentage of samples over/underestimated using the CA test supernatant nD and lab equation as an indicator of IgG by lactation

	Lactation 1 total % of samples			Lactation 2 total % of samples			Lactation 3 or greater total % of samples		
	under - estimated	over - estimated	total	under - estimated	over - estimated	total	under - estimated	over - estimated	total
Actual IgG - LABest[1]									
< 10 mg/ml difference	18.04	21.31	39.35	20.83	15.83	36.66	7.55	9.43	16.98
10 - 20 mg/ml difference	13.11	1.64	14.75	16.25	7.09	23.34	11.32	1.89	13.21
20 - 30 mg/ml difference	16.39	3.28	19.67	10.83	3.33	14.16	11.32	0.00	11.32
> 30 mg/ml difference	16.39	9.84	26.23	12.92	12.92	25.84	30.19	28.30	58.49
TOTAL (%)	63.93	36.07	100	60.83	39.17	100	60.38	39.62	100
Actual IgG -FARMest[1]									
< 10 mg/ml difference	14.75	9.84	24.59	12.92	19.58	32.50	9.44	16.98	26.42
10 - 20 mg/ml difference	11.47	11.48	22.95	13.33	13.33	26.66	3.77	9.44	13.21
20 - 30 mg/ml difference	9.84	14.75	24.59	8.33	9.18	17.51	9.43	13.21	22.64
> 30 mg/ml difference	8.20	19.67	27.87	9.58	13.75	23.33	28.30	9.43	37.73
TOTAL (%)	44.26	55.74	100	44.16	55.84	100	50.94	49.06	100
Actual IgG -WEST									
< 10 mg/ml difference	11.48	29.50	40.98	23.33	22.08	45.41	13.21	20.75	33.96
10 - 20 mg/ml difference	9.84	24.58	34.42	16.25	14.58	30.83	9.43	5.66	15.09
20 - 30 mg/ml difference	3.28	9.84	13.12	5.84	7.50	13.34	15.10	3.77	18.87
> 30 mg/ml difference	3.28	8.20	11.48	5.84	4.58	10.42	26.42	5.66	32.08
TOTAL (%)	27.88	72.12	100	51.26	48.74	100	64.16	35.84	100

Table 16. Percentage of samples over/underestimated using refractive index as an estimate of IgG concentration by storage method

	Fresh total % of samples			Refrgerated total % of samples			Frozen total % of samples		
	under - estimated	over - estimated	total	under - estimated	over - estimated	total	under - estimated	over - estimated	total
Actual IgG - LABest[1]									
< 10 mg/ml difference	25.26	21.98	47.24	15.20	14.62	29.82	16.24	12.47	28.71
10 - 20 mg/ml difference	12.64	8.24	20.88	17.54	12.87	30.41	13.65	7.53	21.18
20 - 30 mg/ml difference	10.44	4.40	14.84	5.85	4.68	10.53	10.59	3.76	14.35
> 30 mg/ml difference	12.64	4.40	17.04	15.20	14.04	29.24	12.47	23.29	35.76
TOTAL (%)	60.98	39.02	100	53.79	46.21	100	52.95	47.05	100
Actual IgG - FARMEST	8.79	20.88	29.67	14.62	18.70	33.32	12.94	16.00	28.94
< 10 mg/ml difference	15.38	14.84	30.22	5.85	18.13	23.98	12.94	15.06	28.00
10 - 20 mg/ml difference	10.44	9.34	19.78	8.19	7.60	15.79	7.06	11.06	18.12
20 - 30 mg/ml difference	6.59	13.74	20.33	14.04	12.87	26.91	9.88	15.06	24.94
> 30 mg/ml difference									
TOTAL (%)	41.2	58.8	100	42.7	57.3	100	42.82	57.18	100
Actual IgG - WEST	19.23	23.63	42.86	23.39	22.80	46.19	18.12	24.47	42.59
< 10 mg/ml difference	13.74	16.48	30.22	9.94	15.79	25.73	9.88	17.88	27.76
10 - 20 mg/ml difference	2.75	9.34	12.09	5.85	7.02	12.87	4.94	9.41	14.35
20 - 30 mg/ml difference	6.04	8.79	14.83	9.36	5.85	15.21	8.48	6.82	15.30
> 30 mg/ml difference									
TOTAL (%)	41.76	58.24	100	48.54	51.46	100	41.42	58.58	100

CHAPTER FOUR

IMPACT OF FREEZE-THAW CYCLES ON THE ACCURACY OF THE CAPRYLIC ACID TEST AND REFRACTOMETRY OF WHOLE COLOSTRUM

ABSTRACT

The objective of this study was to determine the impact of freeze/thaw **(FT)** cycles on the caprylic acid **(CA)** test and whole bovine colostrum **(MC)** refractive index **(nD)** and its relationship to IgG concentration determined by radial immunodiffusion **(RID)**. A total of 797 MC samples collected from 67 dairies across the U.S. were analyzed in this study. Samples had previously been broken down based on storage method (fresh, refrigerated or frozen); this study further divided these groups based on the number of FT cycles prior to analysis by refractometry and RID. Samples that were analyzed by refractometry (CA test or whole MC nD) fresh and went through 1 FT cycle prior to RID analysis resulted in a strong relationship between RID obtained IgG concentration and nD ($r = 0.93$ and 0.90, respectively). The MC samples that were collected fresh, but went through 2 or more FT cycles prior to analysis by refractometry and RID resulted in a weak relationship between RID obtained IgG concentration and nD ($r = 0.09$ and 0.01, respectively). Samples that were refrigerated or frozen prior to collection had weaker relationships between nD and RID for both the whole MC and CA test analysis ($r = 0.38 – 0.80$) regardless of the number of FT cycles. These results indicate that the CA test performed on fresh MC samples provides an accurate and rapid method to determine IgG concentration. The nD of whole MC provides similar results in samples that are analyzed fresh prior to any FT cycles.

Keywords: storage, colostrum, IgG, analysis

INTRODUCTION

Colostral immunoglobulin G **(IgG)** concentration is one of the primary factors affecting passive transfer (Stott and Fellah, 1983; Jaster, 2005). Unfortunately the majority of U.S. dairies do not routinely measure IgG concentration. Based on the 2007 USDA-NAHMS report, only 13% of all U.S. dairy operations evaluate colostrum **(MC)** IgG concentration prior to feeding. Farms that have a herd size of 500 head or more are more likely to evaluate MC IgG concentration (45.2%) as compared to farms with less than 100 head (7.6%). For farms that did measure MC IgG concentration, the most common methods were use of a colostrometer and visual appearance (43.7 and 41.6%, respectively) volume of MC and other methods comprise the remaining 14.7% (NAHMS, 2007).

Previous research in our laboratory has focused on the development and on-farm application of the caprylic acid **(CA)** test and refractive index **(nD)** to rapidly determine MC IgG concentration. Laboratory data suggested that the CA test nD was highly correlated with radial immunodiffusion **(RID)** obtained IgG concentration of MC ($r = 0.96$, $n = 30$ $P < 0.0001$) (Morrill et al., unpublished). Unfortunately the strength of this relationship was not maintained when an on-farm trial was conducted ($r = 0.53$, $n = 805$, $P < 0.001$; Morrill et al., unpublished). Separating the on-farm data into smaller data sets by breed and lactation groups explained some of the differences. Separating the data into groups based on storage method prior to sample collected revealed differences in the relationships between nD and RID. The objective of this study was to determine the impact of freeze/thaw **(FT)** cycles on the CA test and whole MC nD and its relationship to IgG concentration determined by RID.

MATERIALS & METHODS

Eight hundred ninety one MC samples were collected between June and October, 2010. The samples were originally identified as fresh, refrigerated and frozen based on how they were stored prior to feeding. Seven hundred thirty two of these original MC samples were further narrowed down by the number of FT cycles before analysis of nD using refractometry and IgG via RID.

Fresh samples were classified as CAFRESH1 if the sample was collected fresh on the farm, CA test supernatant nD was determined on the farm and the sample went through one FT cycle prior to analysis by RID (n = 112); CAFRESH2 if the sample was collected fresh and went through one FT cycle prior to analysis of CA test supernatant nD and RID (n= 34) or CAFRESH3 iF the sample had an additional FT cycle to re-analyze the sample for IgG by RID, the CA test or whole nD (n = 47). Refrigerated samples were classified as CAFRIDGE1 if the sample was collected from a refrigerated sample; CA test supernatant nD was determined on the farm and the sample went through one FT cycle prior to analysis by RID (n = 75) or CAFRIDGE2 if the sample was from a refrigerated sample and went through one FT cycle prior to analysis of CA test supernatant nD and RID (n = 67). Frozen samples were classified as CAFROZEN1 if the sample was frozen MC that was thawed on the farm, CA test supernatant nD was analyzed on the farm and a second FT cycle occurred prior to RID analysis (n = 83); CAFROZEN2 if the sample was thawed on the farm but had a second FT cycle prior to analysis of both CA test supernatant nD and RID (n = 66) or CAFROZEN3 if the sample was thawed on the farm and had two additional FT cycles prior to analysis of CA test supernatant nD and RID (n = 295). Whole MC samples that were analyzed for the nD were classified in the same manner, but do not have “CA” prior to the group: FRESH1 (n

=29), FRESH2 (n = 128), FRESH3 (n =25), FRIDGE1 (n = 29), FRIDGE2 (n = 123), FROZEN1 (n = 11), FROZEN2 (n = 138), FROZEN3 (n = 296). If there was any question on how many FT cycles a sample went through prior to analysis the sample was deleted from this analysis.

Colostrum Sample Collection

Details on sample collection and analysis of samples for fat, protein, lactose, total solids (TS), other solids (OS) somatic cell count (SCC) coliform and total plate count (TPC) have been previously reported (Morrill et al., unpublished).

Refractometer reading – whole colostrum

A drop of whole MC (~50 µl) was placed on a refractometer prism (SPER SCIENTIFIC model 300034; Scottsdale, AZ) and a brix and nD reading for each sample was recorded. The digital refractometer determines the brix or nD of the liquid being analyzed by shining a light through the sample in the well, measuring the index of refraction and presenting the reading in brix or nD units on a digital scale. The nD is the refractive index of a solution, measured at the wavelength of the sodium D line (589.3 nm) at 20 °C. The Brix value can be obtained from the polynomial fit to the ICUMSA (2009) table: brix = (((((11758.74*nD -88885.21)*nD + 270177.93)*nD - 413145.80)*nD + 318417.95)*nD - 99127.4536).

Caprylic Acid Test Analysis

One ml of MC was added to a tube containing 1.5 ml 0.06 M acetic acid and 75 µl CA. Once the MC was added, samples were mixed for 10 sec and allowed to sit for 60 sec.

After 60 sec the supernatant was removed and analyzed on the digital refractometer for a nD and brix value.

Radial immunodiffusion analysis

Colostrum samples were thawed in a warm water bath and thoroughly mixed prior to RID analysis. One ml of MC was added to 3 ml of distilled water and mixed well. Five µL of diluted colostrum solution was added to each well of a bovine IgG RID test plate (Triple J Farms, Bellingham, WA). Radial immunodiffusion plates were incubated for 24 h and then the diameter of precipitin ring was measured. The diameter of the precipitin ring was compared to a standard curve created by the internal test standards to determine the IgG concentration. All samples were run in duplicate. Samples with a precipitin ring greater than that of the highest internal standard (26.25 mg/ml) were further diluted and re-analyzed. Samples with a precipitin ring smaller than that of the lowest internal standard (1.84 mg/ml) were re-analyzed in an undiluted form.

Statistical Analysis

The Univariate procedure of SAS (SAS Institute Inc., Cary, NC) was used to determine the frequency of observations, as well as to determine outlier samples that would be removed from the data set.

The Proc Corr procedure of SAS (SAS Institute Inc., Cary, NC) was used to determine the relationship between the nD of CA test supernatant or whole MC to the RID obtained IgG concentration. Using the nD measurement from the CA test or whole MC and the correlation to RID obtained IgG concentrations regression equations were developed to estimate the IgG concentration from the nD of the CA test and whole MC based on the storage method.

Epidemiological diagnostic test characteristics (sensitivity, specificity and predictive values) were calculated to compare the digital refractometer nD and IgG estimates to the RID gold standard determined IgG concentration. Sensitivity was defined as the probability of a test result indicative of an inadequate colostral IgG concentration for a sample with an IgG concentration less than 50 mg/ml. Specificity was defined as the probability of a test result indicative of an adequate colostral IgG concentration for a sample with an IgG concentration greater than 50 mg/ml, as determined by means of RID. Predictive values were calculated using the prevalence of samples with an RID IgG concentration of greater than or less than 50 mg/ml and an nD reading above or below the cut point, respectively.

The Proc GLM procedure of SAS (SAS Institute Inc., Cary, NC) was used to determine differences in nutrient composition based on the number of freeze thaw cycles within a storage group according to the model:

$$Y = \mu + F_i + e_1,$$

where Y = the dependent variable; μ = the overall mean; F_1 = the fixed effect of the i^{th} storage level and e_i = the residual error.

The PROC GLM procedure of SAS (SAS Institute Inc., Cary, NC) was used to determine differences nutrient differences across accuracy groups according to the following model:

$$Y = \mu + A_1 + e_1,$$

where Y = the dependent variable; μ = the overall mean; A_1 = the fixed effect of the i^{th} accuracy level and e_i = the residual error.

For GLM models, least square means were determined for each storage and accuracy group. The PDIFF option in SAS (SAS Institute Inc., Cary, NC) was used to separate least square means among storage and accuracy groups; significance was declared at $P \leq 0.005$.

RESULTS

The CAFRESH sample dataset (CAFRESH1 and CAFRESH2) was composed of 116 Holstein samples, three Jersey samples, seven crossbred samples and 20 samples from unidentified cattle. Due to this distribution, breed differences were not able to be determined. This dataset was composed of 30 first lactation samples, 56 second lactation samples, seven third or greater lactation samples and 53 samples from unknown lactations. This dataset was not analyzed for interactions between breed, lactation or pooled samples. CAFRESH3 is identified as its own group and is composed of 35 MC samples from Holsteins, 4 from Jerseys, one from a crossbred and seven from unidentified cattle. Twelve of these samples are from first lactation cattle, 18 from second lactation cattle, two from third or greater lactation cattle and 13 from unknown lactations. The data from refrigerated samples (CAFRIDGE1, CAFRIDGE2, FRIDGE1 and FRIDGE2) is composed of 121 Holstein samples, seven Jersey samples, three crossbred samples and 11 samples from unidentified cattle. Ten of these samples were from first lactation cattle, 36 were from second lactation cattle, 21 samples were from third or greater lactation cattle and 74 samples were from unknown lactations. The CAFROZEN data set (CAFROZEN1, CAFROZEN2 and CAFROZEN3) was composed of 444 MC samples from 195 Holsteins, 68 Jerseys, two crossbreds and 179 unidentified cattle. Fifteen of these samples were from first lactation cattle, 121 were from second lactation cattle, 23 were from third or greater lactation cattle and 285 MC samples were from unknown lactations.

Correlation Coefficients

CAFRESH1 and CAFRESH2 samples had similar relationships between the nD of the CA supernatant and the IgG concentration of MC as determined by RID (Table 1). When the CA test is applied to CAFRESH1 and CAFRESH2 there is a strong relationship (r = 0.93, $P < 0.0001$) between the nD of the CA supernatant and the IgG concentration of the MC sample determined by RID (Figure 1). The relationship between CA quick-test nD and RID decreases for CAFRESH3 samples (r = 0.09, $P = 0.0397$). No additional analysis was completed on the CAFRESH3 samples. Separating CAFRESH1 and CAFRESH2 data based on lactation, breed or if the sample was pooled or obtained from individual cows resulted in similar relationships (Table 2) between nD and IgG determined by RID.

Whole MC FRESH1 samples resulted in a strong relationship between nD and IgG determined by RID (r = 0.90; $P < 0.0001$). FRESH2 samples resulted in a positive relationship between nD and IgG determined by RID (r = 0.78; $P < 0.0001$). Analyzing the nD of fresh, whole MC and comparing it to the IgG determined by RID resulted in an R^2 = 0.80, regardless of if the sample was FRESH1 or FRESH2 (Figure 2). Similar to CAFRESH3 samples, FRESH3 samples resulted in a very poor relationship between nD and actual IgG concentration. No further analyses were conducted on FRESH3 samples.

The correlations between nD of the supernatant and IgG determined by RID for CAFRIDGE1, CAFRIDGE2, CAFROZEN1, CAFROZEN2 or CAFROZEN3 resulted in stronger relationships than those between CAFRESH3 and FRESH3 and IgG but weaker relationships than those between the CA test supernatant nD and actual IgG concentration from CAFRESH1 and CAFRESH2 samples. Correlation differences were not affected by nutrient content (Table 3). CAFRIDGE samples were analyzed for differences in correlations between nD and RID by lactation, breed and pooling (Table 4). Lactation does affect the correlation between nD and RID for CAFRIDGE samples. CAFROZEN samples that were pooled had a strong relationship between nD and actual IgG concentration ($r = 0.87$, $P < 0.0001$; Table 5). CAFROZEN2 samples from Jerseys had a moderate relationship between CA test supernatant nD and actual IgG concentration ($r = 0.79$, $P< 0.001$, $n = 32$), all other breed and CAFROZEN relationships between nD and IgG as determined by RID resulted in correlation coefficients less than 0.50.

The whole MC nD for FRIDGE2 and FROZEN samples had significant relationships with the RID obtained IgG concentration, however these were lower than the relationship between nD and RID for FRESH1 and FRESH2 samples. No breed statistics were calculated for FRESH or FRIDGE samples or FROZEN1 samples. FROZEN2 samples from Holsteins and Jersey's had a strong relationship between nD and IgG determined by RID ($r = 0.83$, $n = 87$ and $r = 76.8$, $n = 36$). FROZEN3 samples from Holsteins and Jerseys both had stronger relationships between nD and IgG determined by RID when analyzed separately ($r = 0.75$, $n = 101$ and $r = 0.86$, $n = 31$, respectivley).

Diagnostic test characteristics

Using the regression equation based on the relationship between the CA test supernatant nD and the actual IgG concentration in fresh MC samples, it was determined that a nD value of 1.34239 was equivalent to 50 mg/ml IgG concentration and a nD value of

1.34228 was equivalent to 49.02 mg/ml IgG concentration. The higher cut-point resulted in a higher PPV and specificity and a lower NPV and sensitivity compared to the lower cut-point (Table 6). Sixteen samples had RID-determined IgG concentrations greater than 50 mg/ml and were classified by the refractive index cut-point of 1.34239 to be less than 50 m/ml. These samples ranged in actual IgG concentration from 50.38 to 64.39 mg/ml. Using the lower cut-point of 1.34228 reduced this number to 12; however the range of actual IgG concentration did not change. Five and six samples had RID determined IgG concentrations less than 50 mg/ml but were classified by the refractometer (both cut-points) to have greater 50 mg/ml. The actual IgG concentration of these samples ranged from 40.60 to 48.14. Samples that are correctly identified as less than 50 mg/ml IgG concentration had a significantly lower percent protein (6.68 vs. 13.19 %), and MUN (15.76 vs. 28.13) and a higher percentage of lactose (3.85 vs. 2.73 %) and OS (3.67 vs. 4.97) compared to samples correctly identified as being greater than 50 mg/ml IgG concentration. There was a trend for samples that contained less than 50 mg/ml IgG to have a higher SCC log (6.33 vs. 5.81) compared to samples with greater than 50 mg/ml IgG. No differences were observed between MC samples contained greater than 50 mg/ml IgG or less than 50 mg/ml IgG for TS, coliform or TPC.

There was a trend ($P < 0.10$) for CAFRESH samples that are incorrectly identified as less than 50 mg/ml IgG concentration to have a higher protein content (10.93 vs 6.68 %) and higher lactose content (3.35 vs. 2.73) compared to samples correctly identified as containing greater than 50 mg/ml IgG. No differences were observed between samples that were incorrectly identified as having IgG concentrations less than 50 mg/ml and samples correctly identified as having an IgG concentration less than 50 mg/ml for fat, OS, TS, MUN, coliform, or TPC. There is not enough nutrient data for the samples that are misidentified having an IgG concentration greater than 50 mg/ml. There is significantly greater coliform growth (2.6 vs 1.38 LOGcfu/ml) in samples that are incorrectly identified as containing greater than 50 mg/ml IgG compared to samples that actually contain greater than 50 mg/ml IgG. No differences in TPC exist between samples misidentified or correctly identified for having greater than 50 mg/ml IgG.

For whole MC samples, FRESH1 and FRESH2 samples were grouped together and compared to the cut-points 1.35966 (Morrill et al., unpublished) and 1.36075 (Table 7). Both cut-points resulted in PPV and NPV greater than 80% and sensitivities greater than 90%. The lower cut-point resulted in a decrease in the specificity of the test to accurately identify MC with an IgG concentration greater than 50 mg/ml.

For CAFRIDGE1 and CAFRIDGE2 samples, a regression equation was created to determine nD cut-points equivalent to 50 mg/ml (1.34032 and 1.34039). When both CAFRIDGE1 and CAFRIDGE2 samples were analyzed the two cut-points resulted in identical PPV, NPV, sensitivities and specificities (Table 8). In this sample set, 105 samples were correctly identified as having IgG concentrations greater than 50 mg/ml, 6 samples were correctly identified as having IgG concentrations less than 50 mg/ml, 30 samples were incorrectly classified as having IgG concentrations greater than 50 mg/ml and 1 sample was incorrectly identified as having an IgG concentration less than 50 mg/ml. The samples that were incorrectly classified as having adequate IgG had actual IgG concentrations ranging from 25.21 to 49.45 mg/ml while the 1 sample that was incorrectly classified as having less than 50 mg/ml IgG had an actual IgG concentration of 51.10 mg/ml.

The two cut-points resulted in identical PPV, NPV, sensitivity and specificity for CAFRIDGE1 samples. The cut-points accurately classifying 49 samples as having IgG concentrations greater than 50 mg/ml and inaccurately classifying 22 samples as having an IgG concentration greater than 50 mg/ml. The MC samples that were misidentified as containing greater than 50 mg/ml IgG had actual IgG concentrations ranging from 33 to 49.45 mg/ml. Three samples were correctly identified as having IgG concentrations less than 50 mg/ml and 1 sample with an actual IgG concentration of 51.100 was misidentified as being less than 50 mg/ml. When CAFRIDGE2 samples were analyzed, the 1.34032 and 1.24039 cut-points correctly identified 56 samples as having IgG concentrations greater than 50 mg/ml and 2 samples as having IgG concentrations less than 50 mg/ml and incorrectly classified 8 samples as having an IgG concentration greater than 50 mg/ml when the actual IgG concentration ranged from 25.21 to 46.20 mg/ml. point.

Cut-points of 1.35162 and 1.35968 were evaluated on whole MC FRIDGEsamples (Table 9). When all FRIDGE samples were evaluated, the lower cut-point resulted in 100% NPV and 100% sensitivity; however 39 samples that had actual IgG concentrations greater than 50 mg/ml were classified by the cut-point as having an IgG concentration less than 50 mg/ml. Additionally the 100% NPV is misleading; only one sample had an nD value less than the 1.35162 cut-point. Samples that had inadequate IgG (less than 50 mg/ml) and were classified as adequate had actual IgG concentrations ranging from 22.48 to 49.45 mg/ml. The higher cut-point provides higher PPV and specificity compared to the lower cut-point for all FRIDGE samples.

Cut-points were created using the regression equations from the three CAFROZEN sample sets. All three cut-points created from the regression equations resulted in identical

PPV, NPV, sensitivity and specificities within the individual sample sets (Table 10). All MC CAFROZEN1 and CAFROZEN2 samples that had greater than 50 mg/ml IgG concentrations were correctly identified by the 3 cut-points; however all samples that had IgG concentrations less than 50 mg/ml were classified as adequate by the cut-points. The actual IgG range for inadequate samples was 16.67 to 49.15 mg/ml for CAFROZEN1 samples and 3.01 to 49.28 mg/ml for CAFROZEN2 samples. CAFROZEN3 samples had similar characteristics as the other sample sets; 67 samples were misidentified as having greater than 50 mg/ml IgG by the cut-points, and one sample (IgG = 53.69) was misidentified as having an IgG concentration less than 50 mg/ml.

Four cut-points were evaluated on frozen whole MC samples, three created from the individual regression equations of each sample set as well as a regression equation encompassing all frozen samples. The cut-points of 1.35935 (Frozen3) and 1.35947 (All frozen samples) resulted in identical PPV, NPV, sensitivity and specificity within FT groups (Table 11). The cut-points of 1.36447 and 1.37748 resulted in high PPV and specificity; however had lower NPV and sensitivities.

Accuracy of refractive index as an indicator of IgG concentration

Using the regression equation based on the relationship between the IgG concentration and nD of CAFRESH samples resulted in 62.33% of the samples being estimated within 10 mg/ml of the IgG concentration as determined by RID assay (Table 12). Samples that resulted in the greatest inaccuracies had IgG concentrations ranging from less than 1.83 to 86.53 mg/ml. While there were IgG mean differences between groups (Table 13), samples that were accurately identified within 10 mg/ml had similar ranges of IgG concentrations (24.64 to 121.22 and less than 1.83 to 125.59 mg/ml) to samples estimated within 10 to 20 mg/ml (40.08 to 105.0 and less than 1.83 to 113.84). Samples that resulted in the greatest inaccuracy had greater fat content compared to all other samples ($P < 0.001$), a lower lactose content than those identified within 10 mg/ml ($P < 0.001$) and a greater TS content compared to those identified within 10 mg/ml ($P < 0.001$). No differences in OS, SCC, coliform or TPC were observed across the accuracy groups.

Using the nD of whole MC, and the regression equation created based on its relationship with RID determined IgG concentration, 53.5% of samples were accurately estimated within 10 mg/ml of the actual IgG concentration (Table 14). Samples that were estimated 30 mg/ml over the actual IgG concentration have a lower IgG concentration compared to samples estimated within 10 mg/ml of the actual IgG concentration (Table 15). No other nutrient differences were detected between the accuracy group within 10 mg/ml and the group estimated over 30 mg/ml of the actual IgG concentration.

For samples that were refrigerated, two regression equations (one from CAFRIDGE1 and one from CAFRIDGE2 samples) were created to estimate IgG concentration from the nD. A greater percentage of CAFRIDGE1 samples were accurately estimated within 10 mg/ml of the actual IgG concentration for both regression equations as compared to CAFRIDGE2 samples (Table16). CAFRIDGE1 and CAfFRIDGE2 samples were at a greater risk to be overestimated than underestimated for both regression equations. Over 60% of CAFRIDGE1 samples were estimated within 20 mg/ml of the actual IgG concentration for both regression equations. Over 45% of CAFRIDGE2 samples were overestimated by more than 20 mg/ml regardless of regression equation utilized. No differences in nutrient composition could be estimated across accuracy groups due to the small numbers of observations per cell. Using the nD of whole MC, the FRIDGE2 regression equation allowed for over 40% of both FRIDGE1 and FRIDGE2 samples to be estimated within 10 mg/ml and nearly 70% were estimated within 20 mg/ml of the actual IgG concentration (Table 17).

Regression equations from the three sets of CAFROZEN samples were used to determine the accuracy of estimating the IgG concentration from the nD of the MC sample. No differences in accuracy were observed with the CAFROZEN3 samples for the three equations across the varying levels of accuracy (Table 18). Over 68% of CAFROZEN1 samples were estimated within 20 mg/ml of the actual IgG concentration using the CAFROZEN1 equation, this percentage decreased for the CAFROZEN2 and CAFROZEN3 equation. Over 68% of CAFROZEN2 samples were estimated within 20 mg/ml of the actual IgG concentration when the CAFROZEN2 or CAFROZEN3 equations were utilized. All three equations created from whole colostrum nD from frozen samples resulted in over 60% of the samples to be estimated within 10 mg/ml of the actual IgG concentration (Table 19). Data presented for FROZEN1 samples should be interpreted with caution as this only includes 11 samples.

DISCUSSION

When samples were analyzed by the CA test with one FT cycle prior to the analysis by RID or the CA quick-test, the correlations coefficients were similar to what was reported in the development of the test in the laboratory (r = 0.96; Morrill et al., unpublished). Whole MC samples that were collected fresh and analyzed by refractometry prior to freezing resulted in a stronger relationship than has been previously reported using brix values (Bielmann et al., 2010; Chigerwe, 2008), however samples that were collected fresh and not analyzed until after one FT cycle resulted in a weaker relationship between nD and RID which was similar to previously reported data on the relationship between nD and RID (Biellman et al., 2010).

Samples that were collected fresh, but had more than one FT cycle prior to analysis, as well as refrigerated and frozen samples resulted in a weaker relationship between nD and RID for both the whole nD and CA test. The process of freezing, storing, thawing and potentially re-freezing can alter biological samples (Farrant, 1980), and result in different SCC (Barkema et al., 1997) estimates and ELISA results (Vanderstichel et al., 2010) as compared to the original, fresh sample. It has previously been reported that the temperature at which milk samples are stored and the thawing process are important for accurate analysis of components, especially SCC and pathogens (Farrant, 1980). Insufficient cell shrinkage during cooling causes the formation of excessive amounts of intracellular ice that may damage cells (Farrant, 1980). Since milk is not a pure solvent that freezes entirely in one step, cells contained within the milk are exposed to extracellular ice, an increased concentration of extracellular solutes and consequently an increased osmolality tending to withdraw water down an osmotic gradient (Farrant, 1980).

Storage and handling of dairy products after harvest and prior to consumption can alter the nutritional and bacterial characteristics. Lipolysis in milk and MC is derived from two enzymatic processes: natural milk lipase (secreted by the animal) and microbial lipases (Antonelli et al., 2002). Natural lipase activity depends on time elapsed between milking and processing or feeding, temperature of storage as well as the handling of milk. When micelles are broken down, the lipids become more available for enzyme attack. Microbial lipases are produced by psychrotrophic bacteria, a microflora that is able to grow in milk during storage at 4^{o} C (Law et al., 1996). Lipoprotein lipase in MC is present bound to the casein micelle and in the milk serum. The unbound lipase is more effective in promoting lipolysis then the casein-bound lipase (Anderson, 1982). If microbial lipases were present in the samples collected for this project, lipolysis may have occurred during storage, and the change in fatty acid composition may have impacted the ability of the acetic acid to bind to the fat, and the CA to precipitate out the non-IgG proteins. Nutrient and bacterial analysis were only completed one each sample once, and individual fatty acids were not analyzed so we cannot definitively say that this is occurring.

While there were no differences in coliform or TPC observed between CAFRESH accuracy groups, the samples that were underestimated by over 30 mg/ml of the actual IgG had greater fat, protein and TS contents and lower lactose content. Whole MC Fresh samples underestimated by greater than 30 mg/ml of the actual IgG also had lower lactose levels, but no differences in fat, protein or total solids. This suggests that the higher concentrations of fat and protein impact the CA test's ability to precipitate out non-IgG proteins, but does not impact the determination of nD of whole MC.

Overall accuracy of estimating IgG concentration from the nD (CA test or whole MC) was optimized when the CA test was performed on fresh samples that were limited to one FT cycle, followed by the whole MC nD of fresh samples that were limited to one FT cycle. Accuracy of refrigerated samples was greatest (45% estimated with 10 mg/ml) for whole MC analysis when refrigerated samples were frozen and analyzed for nD after one FT cycle.

Correlation coefficients for different breeds for fresh and refrigerated samples as well as the impact of pooling for fresh samples should be regarded with caution as these groupings have a small sample number.

To quickly determine if MC is of high enough quality to feed to calves, nD cut-point levels for both the CA test and whole MC need to be determined that strongly correspond to RID values greater than 50 mg/ml. Maximizing the sensitivity of a test allows for the smallest number of samples being inaccurately identified as greater than 50 mg/ml. This prevents the feeding or storage of MC with insufficient IgG (less than 50 mg/ml) thus making sensitivity of a MC quality test more important than specificity. Use of cut-point values lower than optimal would increase the amount of MC classified as adequate, but would increase the chances that inadequate MC would be classified as adequate. Using cut-points greater then optimal, would decreases the chances of inaccurately classifying poor MC as adequate, however some adequate MC may be classified as inadequate.

Diagnostic characteristics were determined based on cut-points for the specific storage method and number of FT cycles, as well as previous suggested cut-points (Morrill et al., unpublished). FRESH3 samples were excluded from the analysis and FRESH1 and FRESH2 samples were combined for both the CA test and whole nD data. The 1.34228 cut-point that is equivalent to 49 mg/ml IgG using the regression equation based on the relationship between CA test nD and RID provided the most beneficial combination of diagnostic characteristics for Fresh samples. The 1.34228 cut-point for fresh samples provided the best combination of diagnostic statistics for the CA test in comparison to other cut-points utilized to analyze refrigerated or frozen samples, again suggesting accuracy of the test is greatest for fresh samples.

Looking at whole MC samples, fresh samples evaluated for two cut-points resulted in similar diagnostic characteristics. Fresh, whole MC samples had a greater sensitivity compared to CA test samples evaluated by the 1.34228 cut-point which is indicative of more whole MC being accurately identified by as having an IgG concentration greater than 50 mg/ml. Lower NPV of fresh, whole MC and higher specificity of the CA test samples indicates that whole MC samples are more likely to be incorrectly classified as above 50 mg/ml, where as the CA test is able to more accurately identify poor MC when the samples are classified as fresh.

CONCLUSION

The objective of this study was to determine the impact of FT cycles on the CA test and whole MC nD and its relationship to IgG concentration determined by RID. The CA test and nD of whole MC, when analyzed fresh, provide rapid and accurate methods to determine IgG concentration. Both test methods have a lower accuracy when samples have been refrigerated or frozen prior to analysis. This study concludes that the nD of fresh, MC provides producers with an easy, rapid and accurate method to determine IgG concentration of MC.

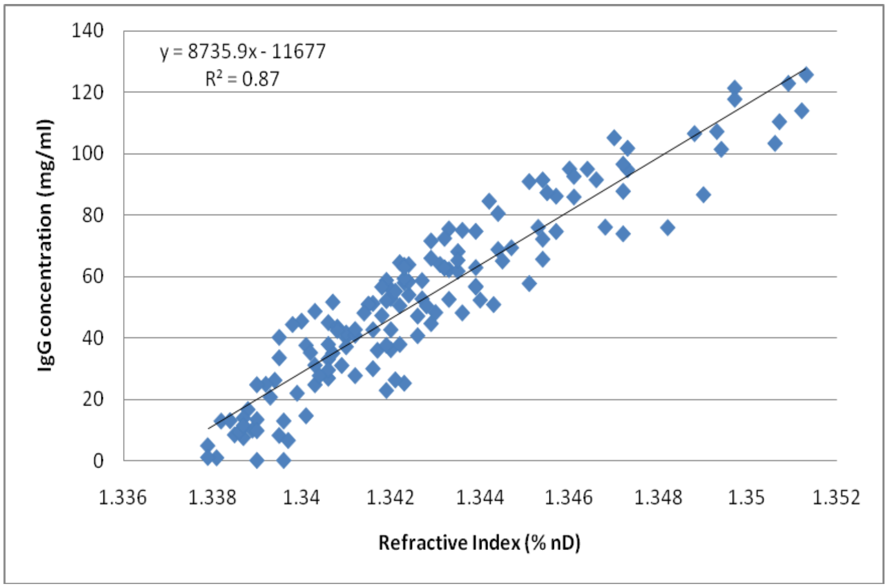


Figure 1. Comparing the refractive index of the CA test to the actual IgG concentration of CAFRESH1 and CAFRESH2 samples (n = 146).

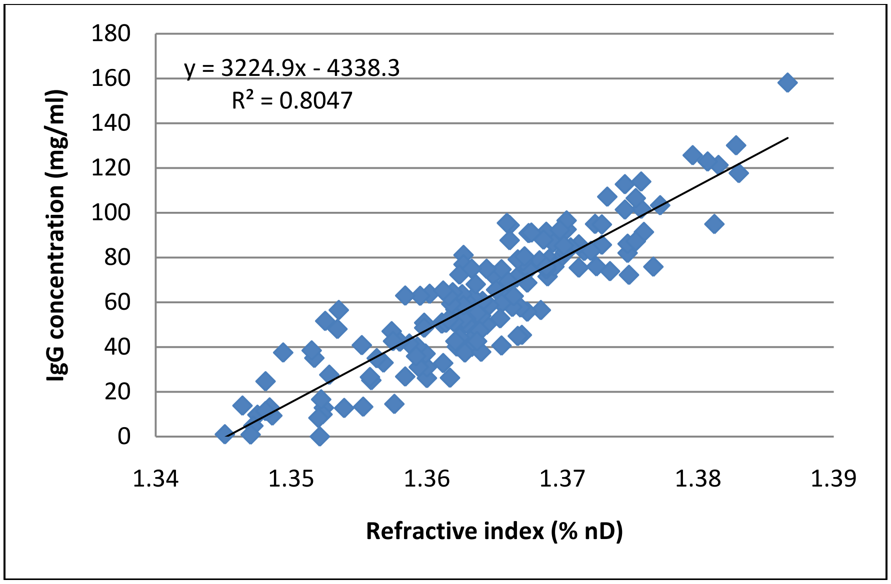


Figure 2. Comparing the refractive index of FRESH1 and FRESH2, whole colostrum samples to the actual IgG concentration (n = 157)

Table 1. Relationship between CA test supernatant nD or whole maternal colostrum nD and actual IgG concentration across storage groups and freeze/thaw cycles.

		RID*nD[1]			RID*WnD[2]	
	n	r	P	n	r	P
CAFRESH1	112	0.93	<0.0001	29	0.90	<0.0001
CAFRESH2	34	0.94	<0.0001	128	0.78	<0.0001
CAFRESH3	47	0.09	0.0397	25	0.01	0.6838
CAFRIDGE1	75	0.43	<0.0001	29	0.41	0.026
CAFRIDGE2	67	0.38	<0.0001	123	0.70	<0.0001
CAFROZEN1	83	0.68	<0.0001	11	0.70	<0.0002
CAFROZEN2	66	0.72	<0.0001	138	0.80	<0.0003
CAFROZEN3	295	0.37	<0.0001	296	0.69	<0.0004
	779			779		

[1] nD = refractive index of CA test supernatant
[2] WnD = refractive index of whole maternal colostrum

Table 2. Correlation coefficients for nD and IgG concentration determined by RID for variants within CAFRESH1 and CAFRESH2 samples

	n	r	P - value
Lactation			
Lactation 1	30	0.93	<0.0001
Lactation 2	56	0.94	<0.0001
Lactation 3+	7	0.97	<0.0000
Unknown lactation	53	0.91	<0.0001
Pooled			
Not pooled	121	0.94	<0.0001
Pooled	11	0.92	<0.0001
Unknown	14	0.89	<0.0001
Breed			
Holstein	116	0.94	<0.0001
Jersey	3	0.99	<0.0001
Crossbred	7	0.92	<0.0001
Unknown	20	0.93	<0.0001

Table 3. Nutrient means of CAFRIDGE1 and CAFRIDGE2 samples

	CAFRIDGE1		CAFRIDGE2	
	Mean	SE	Mean	SE
Nutrient composition				
RID (mg/ml)	63.32	24.04	83.05	32.06
Protein (%)	13.75	3.36	13.47	3.09
Lactose (%)	2.67	0.54	2.73	0.51
Fat (%)	6.18[a]	0.36	4.78[b]	0.34
Other solids (%)	4.19	1.08	4.2	0.6
Total solids (%)	25.48	7.53	22.18	4.86
Coliform (CFU/ml)	349.56	577.71	91.74	209.05
SCC Log	7.88[a]	0.47	5.61[b]	0.4
TPC Log (CFU/ml)	5.91[a]	0.09	5.18[b]	0.09

[ab] Means within a row with different superscripts differ ($P < 0.05$)

Table 4. Correlations coefficients for nD and IgG concentration as determined by RID for sub-groups within CAFRIDGE1 and CAFRIDGE2 samples

	n	r	P - value
Lactation			
Lactation 1	10	0.22	0.5475
Lactation 2	36	0.59	0.0002
Lactation 3+	21	0.09	0.6965
Unknown lactation	74	0.46	<0.0001
Pooled			
Not pooled	83	0.46	<0.0001
Pooled	29	0.5	0.0061
Unknown	29	0.21	0.281
Breed			
Holstein	121	0.47	<0.0001
Jersey	7	0.38	<0.0001
Crossbred	3	0.97	<0.0001
Unknown	11	0.81	<0.0001

Table 5. Correlations coefficients for nD and IgG as determined by RID for sub-groups within CAFROZEN samples

	n	r	P - value
Lactation			
Lactation 1	15	0.51	<0.0001
Lactation 2	121	0.56	<0.0001
Lactation 3+	23	0.69	<0.0001
Unknown lactation	285	0.41	<0.0001
Pooled			
Not pooled	286	0.48	<0.0001
Pooled	22	0.87	<0.0001
Unknown	186	0.38	<0.0001
Breed			
Holstein	195	0.58	<0.0001
Jersey	68	0.5	<0.0001
Crossbred	2	~	~
Unknown	179	0.37	<0.0001

Table 6. Diagnostic test characteristics for the digital refractometer measuring supernatant from the CA test on CAFRESH samples compared with IgG as determined by RID

	Diagnostic Analysis (%)			
Cut-point (%nd)	PPV	NPV	Sensitivity	Specificity
1.33987 [1]	65.84	100	100	38.81
1.34228 [2]	91.78	83.56	84.81	91.04
1.34239 [3]	92.6	79.48	79.74	92.53
1.34242 [4]	92.3	76.54	75.95	92.54

[1] Cut-point suggested from FARMEST equation (Morrill et al., unpublished)

[2] Correlates to IgG concentration of 49 mg/ml using CAFRESH regression equation IgG = 8735.9 * %nD - 11677

[3] Correlates to IgG concentration of 50 mg/ml using CAFRESH regression equation IgG = 8735.9 * %nD - 11677

[4] Cut-point suggested from LABEST equation (Morrill et al., unpublished)

Table 7. Diagnostic test characteristics for the digital refractometer measuring whole maternal colostrum refractive index of FRESH1 and FRESH2 samples compared IgG concentration determined by RID

	Diagnostic Analysis (%)			
Cut-point (%nd)	PPV	NPV	Sensitivity	Specificity
1.36075*	85.58	88.09	94.68	71.1
1.35966**	83.05	89.74	96.08	63.64

*Correlates to IgG concentration of 50 mg/ml using the FRESH regression equation IgG = 3224.9 * nD - 4338.3

** Cut-point suggested by WEST equation (Morrill et al. unpublished)

Table8. Diagnostic test characteristics for the digital refractometer measuring supernatant from the CA test on CAFRIDGE samples compared with the IgG concentration determined by RID.

	All CAFRIDGE Samples Diagnostic Analysis (%)				CAFRIDGE1 samples Diagnostic Analysis (%)				CAFRIDGE2 samples Diagnostic Analysis (%)			
Cut-point (%nd)	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity
1.33987 [1]	87.50	52.10	79.25	66.67	84.21	51.35	64.00	76.00	50.00	14.29	40.00	20.00
1.34032 [2]	76.09	85.57	99.06	15.38	69.00	75.00	98.00	12.00	87.50	100.00	100.00	20.00
1.03039 [3]	76.09	85.57	99.06	15.38	69.01	88.88	98.00	26.67	96.55	11.11	87.50	33.33
1.34242 [4]	76.09	100.00	100.00	8.34	68.06	66.67	98.00	8.00	98.25	10.00	86.15	50.00

[1] Cut-point suggested from LABEST equation (Morrill et al., unpublished)

[2] Correlates to IgG concentration of 50 mg/ml using CAFRIDGE1 regression equation

[3] Correlates to IgG concentration of 50 mg/ml using CAFRIDGE2 regression equation

[4] Cut-point suggested from FARMEST equation (Morrill et al., unpublished)

Table 9. Diagnostic test characteristics for the digital refractometer readings from whole maternal colostrum refrigerated samples compared with the IgG concentration determined by RID.

	All Fridge Samples				FRIDGE1 samples				FRIDGE2 samples			
	Diagnostic Analysis (%)				Diagnostic Analysis (%)				Diagnostic Analysis (%)			
Cut-point (%nd)	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity
FRIDGE1[1]	74.17	100.00	100.00	2.56	75.86	~	100.00	0.00	73.77	100.00	100.00	3.13
FRIDGE2[2]	96.03	86.05	95.28	88.10	84.62	100	100.00	42.86	82.52	75.00	94.44	45.45

[1]Correlates to IgG concentration of 50 mg/ml using FRIDGE1 regression equation

[2]Correlates to IgG concentration of 50 mg/ml using FRIDGE2 regression equation

Table 10. Diagnostic test characteristics for the digital refractometer readings from supernatant from the CA test on CAFROZEN samples compared with the IgG concentration determined by RID.

	CAFROZEN1 Samples Diagnostic Analysis (%)				CAFROZEN2 samples Diagnostic Analysis (%)				CAFROZEN3 samples Diagnostic Analysis (%)			
Cut-point (%nd)	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity
1.33780 [1]	66.27	~	100.00	0.00	68.18	~	100.00	0.00	77.05	66.67	99.56	2.89
1.33987 [2]	85.11	58.33	61.54	75.00	93.55	54.29	64.44	90.47	85.90	50.67	83.63	55.07
1.34093 [3]	66.27	~	100.00	0.00	68.18	~	100.00	0.00	77.05	66.67	99.56	2.89
1.34137 [4]	67.27	~	100.00	0.00	68.18	~	100.00	0.00	77.05	66.67	99.56	2.89
1.34242 [5]	72.00	87.50	98.18	25.00	73.33	83.33	97.78	28.81	77.77	71.43	99.12	7.27

[1] Correlates to IgG concentration of 50 mg/ml using CAFROZEN3 regression equation

[2] Cut-point suggested from LABEST equation (Morrill et al., unpublished)

[3] Correlates to IgG concentration of 50 mg/ml using CAFROZEN1 regression equation

[4] Correlates to IgG concentration of 50 mg/ml using CAFROZEN2 regression equation

[5] Cut-point suggested from FARMEST equation (Morrill et al., unpublished)

Table 11. Diagnostic test characteristics for the digital refractometer readings from FROZEN samples compared with the IgG concentration determined by RID.

Cut-point (%nd)	FROZEN1 samples Diagnostic Analysis (%)				FROZEN2 samples Diagnostic Analysis (%)				FROZEN3 samples Diagnostic Analysis (%)			
	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity
1.35935[1]	50.00	66.67	80.00	33.33	87.50	73.81	88.42	72.09	87.95	82.98	96.48	56.52
1.35947[2]	50.00	66.67	80.00	33.33	87.50	73.81	88.42	72.09	87.95	82.98	96.48	56.52
1.36447[3]	80.00	83.33	80.00	83.33	98.61	63.64	74.74	97.67	96.67	54.31	76.99	91.30
1.37748[4]	~	54.55	100.00	100.00	100.00	33.86	11.58	100.00	100.00	26.04	13.66	100.00

[1] Correlates to 50 mg/ml using the FROZEN3 regression equation

[2] Cut-point suggested by WEST equation (Morrill et al., unpublished)

[3] Correlates to 50 mg/ml using the FROZEN1 regression equation

[4] Correlates to 50 mg/ml using the FROZEN2 regression equation

Table 12. Percentage of samples over/underestimated using the refractive index of CA test supernatant from CAFRESH samples as an indicator of IgG concentration

	Total % of samples[1]		
	Overestimated	Underestimated	Total
Actual IgG - Estimated IgG[2]			
< 10 mg/ml difference	33.56	28.77	62.33
10 - 20 mg/ml difference	19.19	14.38	33.57
20 - 30 mg/ml difference	0	4.10	4.10
Total (%)	52.75	47.25	100

[1] = 146

[2] CAFRESH estimate = %nd * 8735.89922 – 11677

Table 13. Nutrient profile of CA test accuracy groups for CAFRESH samples.

	Within 10 mg/ml		Within 10 - 20 mg/ml		Greater than 20 mg/ml	
	Over	Under	Over	Under	Over	Under
Nutrient						
IgG (mg/ml) [1]	59.12^{b}	41.36^{a}	69.98^{c}	44.74^{a}	~	39.41^{ab}
Fat % [2]	5.43^{a}	4.45^{a}	5.52^{a}	4.63^{a}	~	9.08^{a}
Protein % [2]	10.94^{ab}	9.31^{a}	12.89^{b}	10.77^{ab}	~	13.3^{b}
Lactose % [2]	3.1^{b}	3.51^{c}	2.84^{ab}	3.1^{b}	~	2.28^{a}
Other solids % [3]	3.98	4.31	4.34	4.54	~	3.8
Total solids % [4]	16.25^{a}	16.18^{a}	23.13^{b}	22.59^{b}	~	25.01^{b}
SCC log [4]	6.13	5.93	6.4	6.46	~	5.97
Coliform log [5]	1.59	1.52	1.45	1.28	~	1.09
TPC log [6]	4.87	4.5	4.8	4.84	~	4.68

[1]n = 146
[2]n = 54
[3]n = 58
[4]n = 44
[5]n = 126
[6]n = 137

Table 14. Percentage of samples over/underestimated using fresh whole maternal colostrum refractive index as an indicator of IgG concentration

	Total % of samples[1]		
	Overestimated	Underestimated	Total
Actual IgG - Estimated IgG[2]			
< 10 mg/ml difference	29.29	24.21	53.5
10 - 20 mg/ml difference	14.01	18.47	32.48
20 - 30 mg/ml difference	7.01	7.01	14.02
Total (%)	50.31	49.69	100
Actual IgG - Estimated IgG[3]			
< 10 mg/ml difference	29.29	24.21	53.5
10 - 20 mg/ml difference	14.01	18.47	32.48
20 - 30 mg/ml difference	7.01	7.01	14.02
Total (%)	50.31	49.69	100

[1] n = 157

[2] Estimate = %nd *3224.9- 4338.3

[3] Estimate = %nd* 2975.1 - 3995.1

Table 15. Nutrient profile of whole maternal colostrum fresh accuracy groups

	Within 10 mg/ml		Within 10 - 20 mg/ml		Greater than 20 mg/ml	
	over	under	over	under	over	under
Nutrient					~	
IgG (mg/ml)	69.29^{b}	54.77^{a}	76.89^{b}	43.12^{a}	~	43.48^{a}
Fat %	5.68^{ab}	5.32^{ab}	3.95^{a}	4.40^{ab}	~	7.41^{b}
Protein %	10.93	10.71	10.36	12.19	~	12.4
Lactose %	2.84^{a}	3.30^{a}	3.42^{b}	3.01^{a}	~	2.70^{a}
Other solids %	3.92	4.28	4.15	4.47	~	4.57
Total solids %	19.15	17.21	16.49	20.71	~	23.63
SCC log	5.94	5.71	6.29	6.16	~	6.08
Coliform log	1.16	1.25	1.01	1.36	~	1.14
TPC log	4.7	4.67	4.48	4.46	~	4.98

Table 16. Percentage of samples over/underestimated using the refractive index of CA test supernatant from CAFRIDGE samples as an indicator of IgG concentration

	CAFRIDGE 1 [1]			CAFRIDGE2 [2]		
	Total % of samples			Total % of samples		
	Under-estimated	Over-estimated	Total	Under-estimated	Over-estimated	Total
Actual IgG - Estimated IgG[3]						
< 10 mg/ml difference	14.67	21.33	36.00	7.58	21.21	28.79
10 - 20 mg/ml difference	6.67	25.33	32.00	9.09	15.15	24.24
20 - 30 mg/ml difference	4.00	10.67	14.67	6.06	13.64	19.70
>30 mg/ml difference	13.33	4.00	17.33	16.66	10.61	27.27
Total (%)	38.67	61.33	100.00	39.39	60.61	100.00
Actual IgG - Estimated IgG[4]						
< 10 mg/ml difference	13.34	21.34	34.68	10.61	13.64	24.25
10 - 20 mg/ml difference	8.00	20.00	28.00	4.54	18.18	22.72
20 - 30 mg/ml difference	5.33	17.33	22.66	4.54	13.64	18.18
> 30 mg/m difference	9.33	5.33	14.66	15.15	19.70	34.85
Total (%)	36.00	64.00	100.00	34.84	65.16	100.00

[1] n = 75

[2] n = 66

[3] CAFRIDGE1 = 5800.5 * %nD - 7724.5

[4] CAFRIDGE2 = 7140.9 * %nD - 9521.6

Table 17. Percentage of samples over/underestimated using whole maternal colostrum nD from refrigerated samples as an indicator of IgG concentration

	FRIDGE1 [1]			FRIDGE2 [2]		
	Total % of samples			Total % of samples		
	Under-estimated	Over-estimated	Total	Under-estimated	Over-estimated	Total
Actual IgG - Estimated IgG[3]						
< 10 mg/ml difference	10.35	17.24	27.59	17.06	28.46	45.52
10 - 20 mg/ml difference	13.79	17.24	31.03	10.57	15.45	26.02
20 - 30 mg/ml difference	0	17.24	17.24	4.88	9.76	14.64
>30 mg/ml difference	10.35	13.79	24.14	8.13	5.69	13.82
Total (%)	34.49	65.51	100.00	40.64	59.36	100.00
Actual IgG - Estimated IgG[4]						
< 10 mg/ml difference	27.59	13.79	41.38	21.14	24.39	45.53
10 - 20 mg/ml difference	10.34	17.24	27.58	12.19	13.01	25.20
20 - 30 mg/ml difference	3.45	6.91	10.36	7.32	8.13	15.45
> 30 mg/m difference	10.34	10.34	20.68	11.38	2.44	13.82
Total (%)	51.72	48.28	100.00	52.03	47.97	100.00

[1] n = 29

[2] n = 123

[3] FRIDGE1 = 1385.3x - 1822.4

[4] FRIDGE2 = 3719.4x - 5007.2

Table 18. Percentage of samples over/underestimated using the nD CA test supernatant from CAFROZEN samples as an indicator of IgG concentration.

	CAFROZEN1 [1]			CAFROZEN2 [2]			CAFROZEN3 [3]		
	Total % of samples			Total % of samples			Total % of samples		
	Under-estimated	Over-estimated	Total	Under-estimated	Over-estimated	Total	Under-estimated	Over-estimated	Total
Actual IgG - Estimated IgG[4]									
< 10 mg/ml difference	22.90	25.30	48.20	15.15	24.24	39.39	12.88	12.54	25.42
10 - 20 mg/ml difference	7.23	13.25	20.48	1.52	9.09	10.61	13.22	13.56	26.78
20 - 30 mg/ml difference	9.64	4.82	14.46	4.55	10.61	15.16	9.83	10.85	20.68
>30 mg/ml difference	8.43	8.43	16.86	6.06	28.78	34.84	10.17	16.95	27.12
Total (%)	48.20	51.80	100.00	27.28	72.72	100.00	46.10	53.90	100.00
Actual IgG - Estimated IgG[5]									
< 10 mg/ml difference	16.87	15.66	32.53	19.69	30.30	49.99	14.92	14.58	29.50
10 - 20 mg/ml difference	18.07	12.05	30.12	10.61	9.09	19.70	13.22	9.49	22.71
20 - 30 mg/ml difference	9.64	4.82	14.46	3.03	12.12	15.15	13.90	6.10	20.00
> 30 mg/m difference	18.07	4.82	22.89	10.61	4.55	15.16	19.32	8.47	27.79
Total (%)	62.65	37.35	100.00	43.94	56.06	100.00	61.36	38.64	100.00
Actual IgG - Estimated IgG[6]									
< 10 mg/ml difference	16.87	15.66	32.53	9.09	22.73	31.82	14.58	14.24	28.82
10 - 20 mg/ml difference	9.64	15.66	25.30	3.03	33.33	36.36	12.54	15.25	27.79
20 - 30 mg/ml difference	8.43	14.46	22.89	6.06	6.06	12.12	8.47	9.83	18.30
> 30 mg/m difference	9.64	9.64	19.28	4.55	15.15	19.70	11.19	13.90	25.09
Total (%)	44.58	55.42	100.00	22.73	77.27	100.00	46.78	53.22	100.00

[1] n = 75

[2] n = 66

[3] n = 66

[4] CAFROZEN1 = 6270 * %nD - 8357.6

[5] CAFROZEN2 = 3625.6 * %nD - 4813.3

[6] CAFROZEN3 = 3198 * %nD - 4228.3

Table 19. Percentage of samples over/underestimated using refractive index of frozen samples of whole maternal colostrum as an indicator of IgG concentration

	FROZEN1 [1]			FROZEN2 [2]			FROZEN3 [3]		
	Total % of samples			Total % of samples			Total % of samples		
	Under-estimated	Over-estimated	Total	Under-estimated	Over-estimated	Total	Under-estimated	Over-estimated	Total
Actual IgG - Estimated IgG[4]									
< 10 mg/ml difference	9.09	36.37	45.46	23.91	10.14	34.05	21.62	12.16	33.78
10 - 20 mg/ml difference	27.27	0.00	27.27	20.29	7.97	28.26	20.61	7.43	28.04
20 - 30 mg/ml difference	9.09	9.09	18.18	19.57	0.73	20.30	14.19	1.35	15.54
>30 mg/ml difference	0.00	9.09	9.09	17.39	0.00	17.39	22.64	0.00	22.64
Total (%)	45.45	54.55	100.00	81.16	18.84	100.00	79.06	20.94	100.00
Actual IgG - Estimated IgG[5]									
< 10 mg/ml difference	9.09	18.18	27.27	19.57	27.54	47.11	18.58	26.01	44.59
10 - 20 mg/ml difference	9.09	36.37	45.46	12.32	16.67	28.99	8.78	19.26	28.04
20 - 30 mg/ml difference	0.00	9.09	9.09	9.42	8.70	18.12	3.38	10.14	13.52
> 30 mg/m difference	0.00	18.18	18.18	3.62	2.17	5.79	10.47	3.38	13.85
Total (%)	18.18	81.82	100.00	44.93	55.08	100.01	41.21	58.79	100.00
Actual IgG - Estimated IgG[6]									
< 10 mg/ml difference	9.09	18.18	27.27	18.12	28.26	46.38	18.58	25.00	43.58
10 - 20 mg/ml difference	9.09	36.37	45.46	11.59	15.94	27.53	8.11	20.27	28.38
20 - 30 mg/ml difference	0.00	9.09	9.09	7.97	10.15	18.12	3.72	10.47	14.19
> 30 mg/m difference	0.00	18.18	18.18	4.35	3.62	7.97	9.80	4.05	13.85
Total (%)	18.18	81.82	100.00	42.03	57.97	100.00	40.21	59.79	100.00

[1] n = 11

[2] n = 138

[3] n = 296

[4] Estimate = FROZEN1 equation

[5] Estimate = FROZEN2 equation

[6] Estimate = FROZEN3 equation

CHAPTER FIVE

NATION-WIDE EVALUATION OF QUALITY AND COMPOSITION OF COLOSTRUM ON DAIRY FARMS

ABSTRACT

Objective of this study was to characterize the quality (IgG and nutrient content) of maternal colostrum **(MC)** fed to newborn dairy calves in the United States. Samples of MC (n = 827) were collected immediately prior to feeding from 67 farms in 12 states between June and October, 2010. Samples were collected from Holsteins (n = 494), Jerseys (n =87), crossbred (n = 7) and unidentified cattle (n = 239) from 1st (n = 49), 2nd (n = 174), 3rd and later (n = 128) and unknown (n = 476) lactations. Samples were identified as fresh (n = 196), refrigerated (n = 152) or frozen (n = 479) prior to feeding, as well as if the sample was from an individual cow (n = 734) or pooled (n = 93). Samples of MC were analyzed for IgG by radial immunodiffusion (Triple J Farm; Bellingham, WA), protein, fat, lactose, other solids, total solids and somatic cell count (Dairy Laboratory Service; Dubuque, IA). IgG in MC ranged from < 1 to 200 mg/ml, with a mean IgG concentration of 68.8 mg/ml (SD = 32.8). Thirty percent of MC contained < 50 mg of IgG/ml. IgG concentration increased ($P \leq 0.05$) with parity (42.4, 68.6, 95.9 mg/ml in 1st, 2nd, and 3rd and later lactations, respectively). No differences in IgG were observed across breeds or storage method. Fat content ranged from 1.0 to 21.7% with a mean content of 5.6% (SD = 3.2). Protein ranged from 2.6 to 20.5%, with a mean content of 12.7% (SD = 3.3). Lactose content ranged from 1.2 to 4.5%, with a mean content of 2.9% (SD = 0.5). No nutritional differences were observed across breed;

however fat content was greater ($P \leq 0.05$) in MC from 1st lactation compared to other lactations (6.6, 4.2 and 5.1%, respectively). Lactose and total solids were greater ($P \leq 0.05$) in MC from 1st and 3rd+ lactation cows compared to 2nd lactation cows. Somatic cell count (SCC) ranged from 6,000 to 20,901,000 cells/ml with a mean of 2,531,655 cells/ml. Log SCC decreased ($P \leq 0.05$) from 1st to 2nd and 2nd to 3rd+ lactation MC (5.9, 5.6 and 5.3, respectively). These data suggest that a minimum of 30% of dairy calves in the U.S. are currently fed MC classified below industry standards for IgG content (< 50 mg/ml), and are at a greater risk of failure of passive transfer, mortality and morbidity.

Keywords: colostrum, calves, IgG, nutrients

INTRODUCTION

Fetal development occurs in a protective uterine environment. Immediately after birth the neonate is exposed to a myriad of challenges, including a need for nutrients and protection from microbial pathogens. In utero, the fetal calf obtains nutrients from the placenta. During the third trimester, expression of transporters allows the fetus to obtain carbohydrates **(CHO)**, amino acids **(AA)** and proteins from swallowed amniotic fluid (Buchmiller et al., 1992). Birth represents one of the greatest physiological adaptations; the calf must adjust from obtaining all nutrients from the placenta to intestinal absorption of nutrients received in maternal colostrum **(MC)** and milk. At birth the neonatal intestine has the capability to absorb nutrients along the whole crypt-villus axis, as fetal-type enterocytes are replaced by adult-type epithelial cells, nutrient uptake will be shifted to the upper part of the villus (Pácha et al., 1991). Colostrum provides the neonate with immunoglobulins **(Ig)** essential for passive immunity as well as many other nutrients vital to the calf's survival. The CHO, fat, and protein in MC are essential as metabolic fuels to the newborn (NRC, 2001).

The vitamins and minerals in MC are essential as co-factors for enzymes and general maintenance functions. Increasing evidence in calves and other species indicate that MC also provides maternal leukocytes, growth factors, hormones, cytokines, and nonspecific antimicrobial factors, all of which are necessary to stimulate growth and development of the digestive tract and other organ systems (Davis & Drackley, 1998; Hammon and Blum, 1998; NRC, 2001).

The serum of newborn calves does not contain antibodies or contains only minimal traces of it. Antibodies do not appear in the serum until after the ingestion of MC (Fey, 1971). This is the same phenomenon that occurs in newborn piglets, kids, lambs and foals. In neonatal calves, antibodies from MC are absorbed intact by fetal-type enterocytes and able to be detected in the lymphatics within one to two hours after introduction into the duodenum (Comline, 1951). Antibody uptake by the intestinal epithelial cell occurs within 10 min of exposure (Fey, 1971). In addition to antibodies, the intestinal mucosa of newborn calve non-selectively absorbs all proteins, including *Escherichia coli* **(E. coli)** (Fey, 1971). Colostral IgG have a dual protective role in the neonatal calf (Logan and Penhale, 1971); IgG is absorbed from the small intestine into circulation to protect against septicemia and other immune challenges, IgG that is not absorbed can remain in the intestine and have a local protective effect (Logan and Pearson, 1978).

Immunoglobulin concentration in mature bovine serum and mammary secretions show consistent changes, with the lowest concentration being observed during the second half of lactation, while they increase to 1/3rd higher in the first half of the dry period (Fey, 1971). Immunoglobulin G is transferred from the bloodstream across the mammary barrier by a specific transport mechanism; Fc receptors on the mammary alveolar epithelial cells bind IgG from the extracellular fluid and the molecule undergoes endocytosis, transport and finally release into the luminal secretions (Larson, 1980). Selective transport of IgG_1 into MC requires two separate functions (Larson, 1980). Specific Fc receptors for IgG_1 must be present on the basal plasma membrane of the secretory cells, positioned for binding of the ligand from the extracellular fluid. In addition, mammary epithelial cells must be able to internalize and transcytose IgG_1 in order to deliver it into the luminal secretions (Barrington et al., 2001). Smaller amounts of IgA and IgM are largely derived from local synthesis by plasma-cytes in the mammary gland (Godden, 2008) and a smaller amount transported from the blood (McGuirk and Collins, 2004). During colostrogenesis, homologous and heterologous antibody is concentrated over 100 times more than serum albumin and the immunoglobulin concentration in MC may be about five times that in the serum (of the dam) at parturition (Fey, 1971). During the period of colostrogenesis, up to 500 g/week of IgG are transferred into mammary secretions (Goff and Horst, 1997). The mechanism and regulation of colostrogenesis is not as well known as that of the other stages of mammary gland development. There is clear evidence that colostrogenesis is regulated in part by lactogenic hormones, as estrogen and progesterone are necessary for the initiation of IgG_1 transfer into MC (Barrington, 2001), and is affected by local mechanisms within the mammary gland (Guidry et al., 1979). Colostrogenesis ceases prior to or at the onset of lactation, suggesting that the hormones necessary for lactogenesis are likely candidates for regulating the cessation of IgG transfer (Barrington, 2001).

The majority of the protein composition of MC is IgG (Akers, 2002; Foley and Otterby, 1978), however non–IgG proteins in MC provide nutrition, enhancement of the immune system, act as a defense against pathogenic bacteria, viruses and yeast, and are important for the development of the gastrointestinal tract (Bösze, 2008). The biological properties of proteins in MC facilitate nutrient assimilation and peptides with regulatory activity likely influence the growth and differentiation of various neonatal tissues (Talukder et al., 2001). The effect of these proteins depends on the absorption and transport of colostral macromolecules from the gut of the lumen to specific tissues. Large quantities of AA are also needed for the rapid protein accretion that occurs independent of IgG accumulation in the digestive tract (Davis and Drackley, 1998).

Non-nutritional proteins and peptides, including, but not limited to, cytokines, hormones and growth factors, are present in MC at higher concentrations than found in milk, and potentially modify early gut development (Baumrucker et al., 1993 & 1994; Blätter et al., 2001; Sparks et al., 2003) and immune function. Lactoferrin and other transferrin-like proteins have received attention in both bovine MC and human breast milk as potential bacteriostatic proteins. These proteins bind iron and make it unavailable for bacterial growth (Bezkorovainy, 1977). Lactoferrin in human breast milk has a powerful bacteriostatic effect on E. coli 011/B4 (Bullen et al., 1972). Aside from its antimicrobial activity, it is proposed that lactoferrin plays a role in iron uptake in the intestine and the activation of phagocytes and immune responses (Pakkanen and Aalto, 1997).

The newborn calf is born with relatively small energy reserves, with only 3% of the body weight made of lipids. Much of this lipid content is structural and is unable to contribute to energy needs of the calf. The small amount of fat (380 – 600 g) and glycogen (180 g) in the newborn calf would be mobilized within 18 h of life in the absence of feed intake (Okamoto et al., 1986). Newborn calves rely on lactose and lipids in MC and milk as energy sources. Lactose is hydrolyzed in the small intestine by lactase into glucose and galactose; these sugars are then absorbed by a Na^+ dependent transport pathway, SGLT-1 (Hendriques and Smith, 1974).The activity of this transporter exhibits a proximal to distal gradient, activities of lactase decreases with increasing age and weaning (O'Connor and Diamond, 1999). The gross energy content of MC can be estimated by using the kilocalories available in lactose, non-immunoglobulin protein and fat. Davis and Drackley (1998) calculated the average energy content of MC to be 1.16 kcal/g based on previously reported caloric estimates for lactose, fat and non-Ig protein (Brisson et al., 1957). This is considerably greater than the energy content of milk which is 0.69 kcal/g. The energy content of MC can vary greatly depending on the fat content. The energy provided by fat and lactose in MC is essential for thermogenesis and maintenance of body temperature. Aside from being an energy source, medium chain fatty acids may also play a role in providing antimicrobial protection against viral and bacterial pathogens (Isaacs et al., 1995; Spring et al., 2001).

Over the years, most neonatal calf research has focused on IgG concentration, and improving IgG transport into the intestinal epithelium and absorption into the bloodstream. Many studies have suggested that colostral IgG concentration is the primary factor affecting passive transfer (Nocek et al., 1984), and is the hallmark for evaluation of MC quality. While MC research has primarily focused IgG concentration and recently bacterial contamination, minimal data is available on the nutrient content of MC fed to calves in the U.S. and if MC is providing adequate energy and proteins needed for maintenance and growth.

The objectives of this study were to determine the IgG concentration, nutrient and bacterial composition of MC available on U.S. dairy farms to feed to newborn calves as well as to determine nutrient and bacterial differences in MC across breeds (Holstein & Jersey), parity and storage methods used prior to feeding.

MATERIALS & METHODS

Colostrum Sample Collection

An evaluation of nutrient and bacterial content of bovine MC was performed using 827 MC samples from collected between June and October, 2010 from 67 dairy farms across the United States. Two samples of MC were collected on-farm for analysis to characterize MC available on U.S. dairy farms. Samples were frozen, placed on dry ice and shipped to the respective lab for analysis. Samples were classified based on their storage method prior to feeding as fresh (no storage), refrigerated or frozen. Additional information including breed, lactation number, milking number post-calving and if the sample was from an individual cow or if it was from a pooled source were recorded for each sample.

Colostrometer reading

A bovine colostrometer (Nasco; Fort Atkinson, WI) was placed in a graduated cylinder containing MC. A reading in mg/ml was recorded at the point of bouyancy.

Radial immunodiffusion analysis

Colostrum samples were thawed in a warm water bath and thoroughly mixed prior to RID analysis. One ml of MC was added to 3 ml of distilled water and mixed well. Five µL of diluted colostrum solution was added to each well of a bovine IgG RID test plate (Triple J Farms, Bellingham, WA). Radial immunodiffusion **(RID)** plates were incubated for 24 h and then the diameter of precipitin ring was measured. The diameter of the precipitin ring was compared to a standard curve created by the internal test standards to determine the IgG concentration. All MC samples were run in duplicate. Samples with a precipitin ring greater than that of the highest internal standard (26.25 mg/ml) were further diluted and re-analyzed.

Samples with a precipitin ring smaller than that of the lowest internal standard (1.84 mg/ml) were re-analyzed in an undiluted form.

Nutrient and bacterial analysis

The second sample was sent to the DHI laboratory (Dubuque, IA) for nutrient and bacterial analysis. The components were analyzed on a FOSS Milkoscan FT+ and the SCC was analyzed on a FOSS Fossomatic FC. Plate counts were performed with the Petrifilm Plate Loop Count method as outlined in section 6.030 of the 17th Edition of the Standard Methods for the Examination of Dairy Products. Coliform counts were performed with the Petrifilm Coliform Count Plate Method (SMEDP 7.071).

Statistical Analysis

The Univariate procedure of SAS (SAS Institute Inc., Cary, NC) was used to determine the frequency of observations, as well as to determine outlier samples that would be removed from the data set.

IgG concentration, nutrient and bacterial data from MC samples were analyzed using the PROC GLM procedure of SAS (SAS Institute Inc., Cary, NC) according to the model:

$$Y = \mu + La_o + B_p + St_q + LaB_{oq} + e_{opq}$$

where Y = the dependent variable; μ = the overall mean; La= the fixed effect of the o^{th} lactation (m =1,…7); B = the p^{th} breed (p = Holstein, Jersey, crossbred); St = the fixed effect of q^{th} storage (q = fresh, refrigerated, frozen); LaB = the fixed effect interaction between o^{th} lactation and p^{th} breed; and e = the residual error.

Correlations between IgG, nutrient and bacterial composition were determined using the PROC CORR procedure of SAS. Statistical significance was declared when $P \leq 0.05$.

RESULTS

Descriptive Analysis

A total of 827 MC samples from the original dataset were analyzed for this study (Table 1). These samples were collected from 67 farms across 12 states. Samples were collected from 734 individual cows, and 93 MC pools. Samples were obtained from 494 Holsteins, 87 Jerseys, seven crossbreds and 239 unidentified cattle. This encompassed samples from 49 first lactations cows, 174 second lactations cows, and 128 third or greater lactation cows. The majority of the MC samples collected represent the first milking after calving, and were frozen prior to feeding.

The overall sample means as well as breed, lactation and storage means for IgG concentration, nutrient and bacterial composition are presented in Table 2. The IgG concentration of MC samples ranged from < 1.84 mg/ml to 200.18 mg/ml (Figure 1). The overall mean IgG concentration of the samples analyzed was 69.34 mg/ml (SD = 32.8). IgG concentration increased ($P \leq 0.05$) with parity (42.4, 68.6, 95.9 mg/ml in first, second, and third or later lactations, respectively). No differences in IgG concentration were observed across breeds or storage methods. Samples from crossbred cattle were not included in breed differences due to the small sample size. Samples that were from individual cows and not pooled had a greater IgG concentration compared to pooled samples (Table 3).

Across the 827 MC samples, fat content ranged from 1.0 to 21.7% with a mean fat content of 5.6% (SD = 3.2), protein ranged from 2.6 to 20.5%, with a mean protein content of 12.7% (SD = 3.3), lactose content ranged from 1.2 to 4.5%, with a mean lactose content of 2.9% (SD = 0.5). No nutritional differences were observed across breed, however, nutrient differences were observed across parity and storage groups. Fat content was greatest ($P \leq 0.05$) in MC from first lactation cattle compared to other lactations, and lowest in samples from second lactation cattle. Not fat differences were observed in storage groups or if the sample was pooled. Protein content was higher in refrigerated samples ($P < 0.05$) and lowest in fresh samples ($P < 0.05$). Protein content was also higher in individual samples as compared to pooled MC samples. Lactose was higher in samples collected from cattle in their third or greater lactation ($P < 0.05$) compared to samples collected from first lactation cattle and was also greater in fresh samples compared to refrigerated or frozen samples. Other solids and TS were higher in samples collected from cattle in their first or third and greater lactation samples compared to samples from second lactation cattle and highest in refrigerated samples. Total solids were greater in individual samples as compared to pooled MC samples.

Somatic cell count ranged from 6,000 to 20,901,000 cells/ml with a mean of 2,531,655 cells/ml. Log SCC decreased ($P \leq 0.05$) from first to second and second to third and greater lactation MC (5.9, 5.6 and 5.3, respectively). The TPC ranged from less than 10 to 5,900,000 cfu/ml, with a mean of 554,020 cfu/ml. Coliform counts ranged from less than 10 to 1,500 cfu/ml with a mean of 120.86 cfu/ml (SD = 328). The MC samples collected from Holsteins had higher SCC, coliform counts and TPC compared to MC samples collected from Jerseys. Samples collected from first lactation cattle had the highest SCC count, while samples collected from 3rd and greater lactation cattle had the lowest SCC (P < 0.01). Coliform counts were highest in samples collected from second lactation cattle. Samples of MC that were refrigerated prior to feeding had the highest TPC and coliform counts compared to fresh and refrigerated samples, while fresh and frozen samples had higher SCC compared to refrigerated samples. Coliform and TPC were highest in pooled samples as compared to individual MC samples.

Correlations between nutrients in maternal colostrum

The IgG concentration of MC was moderately correlated with protein (r = 0.69, P < 0.0001, n = 555), the relationship was strongest in first and second lactation samples (r = 0.86, n = 27 and r = 0.77, n = 132). A moderate correlation between RID and TS (r = 0.41, P < 0.001, n = 509) was observed in the overall dataset, this was also stronger in first lactation (r = 0.73, n = 25, P < 0.0001), second lactation (r = 0.61, n = 131, P < 0.0001) as well as in fresh (r = 0.71, n = 51, P < 0.0001) samples. The IgG concentration in MC was moderately correlated with MUN (r = 0.41, P < 0.0001, n = 530) for the overall dataset with a stronger relationship for first lactation (r = 0.75, n = 26, P < 0.0001) and third and greater lactations (r = 0.67, n = 46, P < 0.0001). The IgG concentration was negatively correlated with lactose (r = -0.48, P < 0.0001, n = 551), this relationship was strongest in samples from first lactation (r = -0.78, n = 27, P < 0.0001).

The colostrometer estimate of IgG was moderately correlated with MUN (r = 0.43, P < 0.002, n = 72) and had a moderate negative relationship with TPC (r = -0.42, P < 0.002, n = 75); these relationships were similar across breeds, lactation and storage methods. Total solids were correlated with protein (r = 0.71, *P* < 0.0001, n =545), fat (r = 0.67, *P* < 0.0001, n = 534), MUN (0.57, *P* < 0.0001, n = 546) and moderately negatively correlated with lactose (r = -0.37, *P* < 0.0001, n = 540). The relationship between TS and protein was stronger in first lactation samples (r = 0.87, n = 25, *P* < 0.0001) and third and greater lactation samples (r = 0.90, n = 44, *P* < 0.0001). Other solids were highly correlated with lactose (r = 0.81, *P* < 0.0001, n = 588). Protein was correlated with MUN (r = 0.67, *P* < 0.0001, n = 545) and negatively correlated with lactose (r = -0.61, *P* < 0.0001, n = 588).

DISCUSSION

The data presented in this paper represents MC that was available on farms during the months of June through October, 2010, and is not necessarily representative of the MC produced by dairy cattle in the United States. Samples were collected from farms that were willing to allow for the collection of MC, were not pasteurizing or adding a preservative to MC and were also willing to complete a management survey for an additional study.

IgG and nutritional composition

The nutrient and IgG composition of MC changes throughout the first six milkings postpartum (Foley and Otterby, 1978) and can be influenced by breed, parity (Muller and Ellinger, 1981; Kume and Tanabe, 1993; Gulliksen, 2008), dry period length and time of milking post partum. In a study done in Norway, 13.7% of total variation in MC quality could be explained by cluster effects within an individual herd (Gulliksen et al., 2008). Mean IgG concentration of samples collected was similar to previous reported data (68.5 $\pm$ 32.4 mg/ml; Chigerwe et al., 2008), but lower than that recently presented by Bielmann et al. (2010; 94.4 mg/ml). Current industry recommendations include discarding MC with less than

50 mg/ml IgG; 29.38% of the samples collected in this study had IgG concentrations less the recommended IgG level (Table 4), thus potentially putting nearly 30% of U.S. calves at risk of failure of passive transfer **(FPT)**. When grouped by breed, lactation, and storage method, distribution of IgG was similar; however, pooled samples had a slightly greater percentage of inadequate samples (35.48%). This percentage of samples is lower than the 57.8% reported in a study that represented MC quality in Norwegian dairy cattle (Gulliksen et al., 2008). Similar to previous research, IgG concentration increased in MC as parity increased (Devery-Pocius and Larson, 1983; Donovan et al., 1986). It has been suggested that the increase in IgG concentration is due to an increase in antigenic exposure and incidence of disease (Jensen, 1975; Donovan et al., 1986). Colostrum production is often lower in first lactation cattle, suggesting less mammary development, and potentially reduced transport capacity for IgG into the mammary gland (Devery-Pocius and Larson, 1983).

Muller and Ellinger (1981) compared total IgG content of MC among various breeds of dairy cattle; it was reported that Holsteins produced MC with total Ig content (5.6%) that was numerically lower than for Guernsey (6.3%) and Brown Swiss (6.6%), and was statistically lower than Ayrshire (8.1%) and Jerseys (9.0%). It was concluded that these differences could be attributed to genetic differences and/or dilution effects. While our study did not observe breed differences in mean IgG concentration, MC with inadequate IgG may have been discarded and therefore only high quality MC was available for collection. This could also explain why no differences were observed in mean IgG concentration based on storage method.

The mean fat content of the entire sample set was slightly lower (5.6 vs. 6.7%) as compared to that cited by Foley and Otterby (1978) and greater than that reported by Fleenor and Stott (1980); Quigley et al. (1994) and Tsioulpas et al. (2007). The sample set used by Fleenor and Stott consisted of only 14 Holstein-Friesian cows, the sample set used by Tsiulpas consisited of only eight Freisians, while the sample set used by Quigley et al. (1994) consisted of 49 Jersey cows. Similar to the overall mean, the Jersey samples in our data had a greater fat content (5.25% compared to 4.00%, respectively) then that reported by Quigley et al. (1994). Similar to the Quigley data set, a large variation of fat content in MC was observed in this study (1.02 to 21.69%). A lower fat content of MC should be a concern to producers as well as researchers, as newborn calves are born with limited fat (380 to 600g) and glycogen (180 g) reserves (Okamoto et al., 1986) and calves less than 10 d of age exhibit high rates of metabolism (Roy et al., 1957). Fat is the most energy dense component of MC, with a caloric value of 9.11kcal/g (Brisson et al., 1957). It is estimated that the metabolizable energy requirement of calves falls between 90 and 110 kcals of ME/kg$^{0.75}$ (Davis and Drakley, 1998). The NRC (2001) sets the net energy for maintenance requirement at 0.0086 Mcal/kg$^{0.75}$ of live weight. Using the mean fat content of the overall data set of 5.61%, the energy from fat is only 0.511 kcal/g of MC; this is lower than would be expected using the 6.7% fat reported by Foley and Otterby (1978), which results in 0.61 kcal/g of MC. Both calculated energy values are much greater than the energy provided by milk with a 3.6% fat content (0.33 kcal/g). This suggests that feeding MC beyond day 1 could provide additional energy to newborn calves compared to whole milk; this may be greatly beneficial during winter months. Additionally, this suggests that MC from first lactation cattle, with a lower IgG concentration may still be beneficial and could be stored to be fed on day 2 or 3 of life as an energy source, as fat content was greatest (6.55%) in first lactation samples and there was not a correlation between IgG and fat content.

Colostrum contains nearly double the amount of TS as compared to milk (Foley and Otterby, 1978), this amount of TS is primarily due to the high amounts of IgG and overall greater protein content. Lactose is the primary CHO in MC and milk providing 3.95 kcal/g (Brisson et al., 1957). During the first three milkings postpartum the lactose content increases, 2.7, 3.9 and 4.4%, respectively (Foley and Otterby, 1978). Similar findings were more recently reported (Tsioulpas et al., 2007), this study observed that lactose continues to increase until 30 d postpartum (4.54% $\pm$ 0.21). Feeding MC or milk stimulates the development of lactase activity in the jejunum of newborn piglet (Burrin et al., 1994; Tivey et al., 1994) and MC also leads to a greater jejunal mass and protein content at 6 and 24 h as compared to un-fed, water-fed or milk-fed piglets (Burrin et al., 1994). Tivey et al. (1994) also reported that enterocyte lactase-phlorizin hydrolase **(LPH)** activity can be negatively influenced by the absence of lipids in MC fed to newborn piglets and that glucose, not galactose, is important in mediating the increase in LPH activity during the first day of postnatal life. These early influences on LPH activity moderated by MC consumption are essential for the efficient digestion of lactose during the pre-ruminant phase of calves.

The third component of MC that can be an energy source, but is more important as an AA source is protein. The high rate of tissue protein turnover in young calves may be responsible for the low efficiency of use of metabolizable energy for protein gain (Geay, 1984). Colostrum contains greater total protein as compared to milk (14.0 vs 3.2%) and a greater percentage of IgG (6.0 vs 0.09%; Foley and Otterby, 1978). Total protein decreases rapidly from 1 d postpartum (16.12 $\pm$ 1.82) to 2 d post-partum (5.43 $\pm$ 0.24) and continues to decrease until 30 d postpartum (3.08 $\pm$ 0.19; Tsioulpas et al., 2007). Non-Ig protein potentially provides 5.60 kcal/g (Brisson et al., 1957) as well as providing essential AA needed for protein accretion. Using neonatal lambs as a model, 35% of the dietary AA ingested in MC was available within 4 h for AA metabolism, AA were provided by β-lactalbumin, casein and IgG_1 (Yvon et al., 1993), and free AA concentrations in the blood increased in MC fed lambs (280 $\pm$ 20 vs 369 $\pm$ 15 mg/L) as compared to milk fed lambs (Yvon et al., 1993). It was also observed that whey proteins (including IgG) were minimally hydrolyzed and rapidly drained from the abomasum. The contribution of AA from MC is dependent on three factors: volume fed, gastric emptying and gastrointestinal degradation.

It has previously reported that protein content was lowest in first lactation cattle, and increased in second and third lactation cattle (15.1, 16.9 and 18.8%, respectively), this data was collected from Holstein cattle (n =21) in Japan (Kume and Tanabe, 1993). Our study did not observe any parity or breed differences in protein content, but did observe differences in protein content by the storage method. Foley and Otterby (1978) previously reported that storage of MC at ambient temperatures leads to a decrease in pH, TS, protein, fat and lactose, while freezing resulted in virtually no nutrient loss. While we cannot comment on potential nutrient alterations based on storage method, it was observed that protein was greater in refrigerated and frozen samples compared to fresh samples, TS was greater in refrigerated samples and lactose was greatest in fresh samples.

While MC provides important antibodies and nutrients essential for survival, it can also serve as an initial source of pathogens. In addition to discarding MC containing less than 50 mg/ml IgG, it is also advised to discard MC that is bloody, watery, from cows with mastitis, contains a TPC greater than 100,000 cfu/ml (McGuirk and Collins, 2004) or is from a cow that is known to carry leukosis or Johnes disease. New mastitis infections often occur during the dry period, and this is an area that affects MC quality. Gulliksen et al. (2008) reported that a somatic cell count of greater than 50,000 cells/mL was the only test-day result found to be significant for the production of MC with very low IgG values (less than 30 mg/ml). Our study did detect a weak negative relationship between SCC and IgG (r = -0.27, n = 561, $P < 0.0001$) and all MC samples with IgG concentrations less than 25 mg/ml had SCC greater than 400,000. This relationship was slightly stronger in samples collected from Holsteins (r = -0.31, n = 283, $P < 0.0001$), third and greater lactation cattle (r = -0.42, n = 48, $P = 0.0027$) and refrigerated (r = -0.44, n = 112, $P < 0.0001$) samples. The greatest SCC was observed in MC samples collected from first lactation cattle.

Calving pens represent a great threat to newborn calves, as exposure to *Escherichia* E. coli prior to ingestion of MC can be detrimental to the health status of calves (Corley et al., 1977). Bacteria in MC may bind free IgG in the gut lumen or potentially block uptake of IgG molecules across intestinal epithelial cells (James, 1978; James 1981) and potentially limit the calf's ability to obtain adequate passive transfer. Johnson et al., (2007) demonstrated reduced uptake of IgG, with newborn calves being fed 3.8 L of pasteurized MC (813 cfu/ml) or 3.8 L raw MC (40,738 cfu/ml). Calves receiving the MC with lower bacterial contamination had greater 24 h serum IgG concentrations compared to their counterparts (22.3 and 18.1 mg/ml, respectively). While both groups of calves obtained adequate passive transfer, the apparent efficiency of absorption of IgG absorption was reduced in calves fed MC with greater bacterial contamination (33 vs. 27%).

Our study observed that total coliform counts were lower in Jerseys compared to Holsteins. This is potentially due to farm management, as MC samples from Jerseys came from fewer farms. Samples of MC that were stored in a refrigerator had greater coliform counts compared to fresh or frozen samples. It is currently recommended to feed MC that contains fewer than 10,000 cfu/ml total coliform count (McGuirk and Collins, 2004). The MC collected from this study was well below the recommended upper limit.

Over 50% of the MC samples collected in our study had TPC less than 100,000 cfu/ml (Table 5), however over 27% of the samples collected had TPC greater than 500,000 cfu/ml. This introduces a huge immunological challenge to newborn calves who are not equipped to handle a large pathogen load. A greater percentage of MC collected from Jerseys fell below the 100,000 cfu/ml cut-point compared to MC collected from Holsteins (Table 6), this could potentially be due to individual herd management. Over 60% of the samples that were fresh or frozen prior to sample collection had TPC below 100,000 cfu/ml, while over 40% of refrigerated samples had TPC higher than 1 million cfu/ml (Table 7).

Stewart et al. (2005) reported that significant bacterial contamination occurred during the process of milking and handling of MC prior to feeding and that bacteria can rapidly multiply if MC is stored at warm temperatures. A stipulation for a farm to participate in this study was that they did not pasteurize or add a preservative to MC prior to storage or feeding. Based on the data presented it here, it could be suggested that more farms should investigate the option of pasteurization of MC to reduce the pathogen load presented to calves during feeding.

Storage method impacts MC quality by altering bacterial growth and shelf life. Previous research has observed that refrigeration slows pathogen growth compared to unrefrigerated samples, if MC is stored for 24 h (Stewart et al., 2005). Current recommendations are for MC to be refrigerated if fed within 24 h of collection and frozen if it is going to be stored for more than 24 h (BAMN, 2001). The 2007 NAHMS report observed that 60.7% of MC being hand-fed was fed fresh or stored without refrigeration, 11.1% was refrigerated and 28.2% was frozen prior to feeding. Data from our study suggests an industry shift towards an increase in freezing MC (57.9%) as compared to feeding refrigerated (18.4%) or fresh (23.7%) MC.

Combining the industry recommendations for adequate IgG (greater than 50 mg/ml) and low TPC (less than 100,000 cfu/ml), only 39.41% of the MC samples collected for this study were acceptable (Table 8), over 30% of our MC samples met the IgG requirement but exceeded the TPC requirement, and additional 15% of samples met the TPC requirement but had inadequate IgG concentration. The breakdown of individual MC samples that met both industry standards was similar to the overall set, however pooled samples had a slightly greater percentage of samples that met the IgG requirement but not the TPC requirement. A greater percentage of Jersey samples met both industry recommendations (63.51%) as compared to samples from Holsteins. Over 40% of fresh and frozen samples met both recommendations, however only 18.52% of refrigerated samples met both requirements. Over 50% of refrigerated samples met the IgG requirement but had TPC greater than 100,000 cfu/ml. Our laboratory is currently analyzing survey data that was collected on each MC sample with the goal of identifying key management practices that impact colostral quality.

CONCLUSIONS

The objectives of this study were to characterize the the IgG concentration, bacterial contamination and nutrient composition of MC available on U.S. dairy operations. The overall nutrient composition of MC from our dataset was not dramatically different from other reports over the past 30 years. No breed differences in nutrient composition were observed however lactational differences suggest that the energy content of MC may be greater first lactation animals, suggesting this MC could be saved and fed at the second or third feeding as an energy source. Storage method of MC has a significant impact on bacterial contamination, based on this data set, MC should be fed fresh or frozen immediately and not stored in a refrigerater. This study observed IgG concentration in MC ranging from < 1.84 to 200.18 mg/m; of the 827 sample collected 29.38% did not meet the industry standard for containing greater than 50 mg of IgG/ml. Bacterial contamination of MC continues to be a problem on many farms, only 54.8% of the samples obtained for this study had TPC less than the industry recommendation of 100,000 cfu/ml. When both of these industry standards for MC quality were combined only 39.41% percent of samples were adequate. Data collected fro, this study indicated that a large percentage of calves are being put at risk for FPT, as well as pathogen exposure.

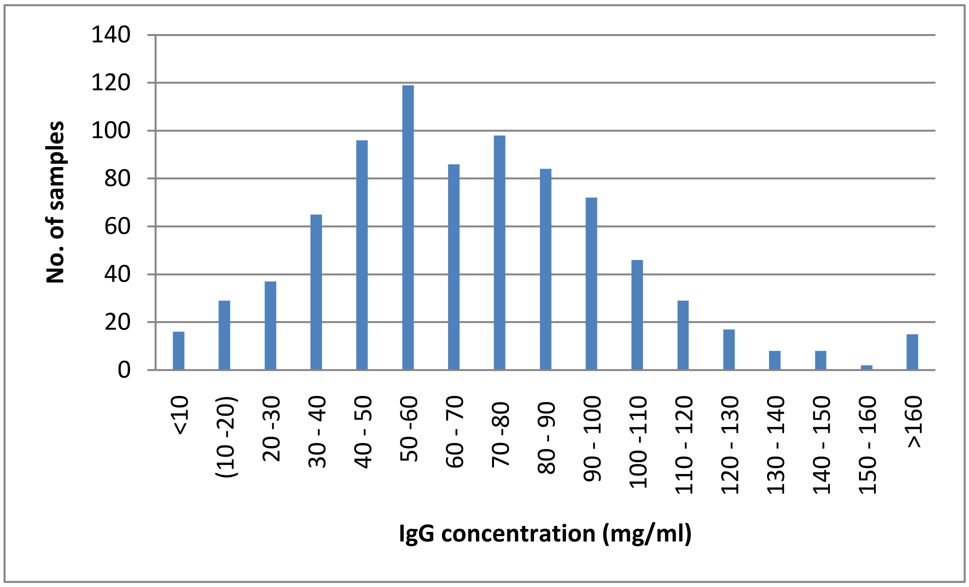


Figure 1. A distribution plot of the IgG concentration of 827 colostrum samples.

Table 1. Colostrum samples collected across state, breed, lactation number, milking post calving and storage method

			Breed				Lactation					Milking post calving			Stored[1]		
State	Farms	Samples	Holstein	Jersey	Crossbred	N/A	1	2	3	4+	N/A	1	2	3	1	2	3
AZ	2	61	40	~	~	21	2	7	~	~	52	61	~	~	7	9	45
CA	14	173	161	8	~	4	34	76	~	~	63	169	4	~	93	34	46
FL	4	35	33	2	~	~	1	22	~	~	12	30	~	~	17	9	9
GA	2	30	30	~	~	~	~	30	~	~	~	30	~	~	11	19	~
IA	1	40	40	~	~	~	~		~	~	40	40	~	~	~	~	40
MN	11	97	35	~	~	62	8	10	4	5	70	97	~	~	2	6	89
NH	1	18	18	~	~	~	~	6	3	9	~	6	6	6	18	~	~
NY	5	59	41	3	~	15	4	11	4	2	38	49	5	5	17	29	13
PA	5	51	42	9	~	~	~	5	17	22	7	46	4	1	~	23	28
TX	9	176	5	62	7	102	~		61	~	115	175	1	~	6	3	167
VA	7	60	22	3		35	~	2	1	~	57	60	~	~	25	20	15
WI	6	27	27	~	~	~	~	5	~	~	22	26	1	~	~	~	27
TOTAL	**67**	**827**	**494**	**87**	**7**	**239**	**49**	**174**	**90**	**38**	**476**	**789**	**21**	**12**	**196**	**152**	**479**

[1] 1 = fresh (not stored), 2 = refrigerated, 3 = frozen

Table 2. Nutrient and bacterial means by breed, lactation and storage

	Overall mean	Breed			Lactation				Stored[1]			
		Holstein	Jersey	SE	1	2	3	SE	1	2	3	SE
IgG (mg/ml)	68.84	74.16	65.77	8.33	42.39^{c}	68.57^{b}	95.87^{c}	9.3	69.04	74.55	66.31	7.34
Fat (%)	5.61	5.33	5.25	0.50	6.55^{c}	4.2^{a}	5.14^{b}	0.53	4.88	5.37	5.64	0.47
Protein (%)	12.72	12.47	12.59	0.67	12.35	12.09	13.14	0.73	10.92^{a}	14.1^{c}	12.55^{b}	0.64
Lactose (%)	2.9	2.97	2.93	0.10	2.99^{ab}	2.78^{a}	3.08^{b}	0.10	3.18^{b}	2.75^{a}	2.92^{a}	0.09
Other Solids (%)	4.34	4.44	4.40	0.08	4.43^{b}	4.24^{a}	4.59^{b}	0.08	4.56^{b}	4.31^{a}	4.38 ^{a}	0.07
Total Solids (%)	22.56	22.15	22.98	0.90	23.46^{b}	20.83^{a}	23.40^{b}	0.99	21.21^{a}	24.16^{b}	22.33^{a}	0.87
SCC Log	5.86	5.89^{b}	5.33^{a}	0.13	5.99^{c}	5.59^{b}	5.26^{a}	0.15	5.79^{b}	5.46^{a}	5.58^{b}	0.13
Coliform Log	1.33	1.53^{b}	1.16^{a}	0.14	1.24^{a}	1.54^{b}	1.26^{a}	0.13	1.12^{a}	1.58^{b}	1.34^{a}	0.14
TPC Log	4.96	4.88^{b}	4.11^{a}	0.14	4.49^{ab}	4.70^{b}	4.31^{a}	0.14	3.97^{a}	4.99^{c}	4.54^{b}	0.13

[1] 1 = fresh (not stored), 2 = refrigerated, 3 = frozen

[abc] Differences between means are indicated by different alphabetical superscripts ($P < 0.05$)

TABLE 3. Nutrient and bacterial means of individual samples compared to pooled samples of maternal colostrum

	Pooled			p-value
	NO	YES	SE	
	n = 734	n = 93		
IgG (mg/ml)	69.65^{b}	60.25^{a}	3.64	0.0145
Fat (%)	5.73	6.13	0.57	0.5026
Protein (%)	13.21^{b}	10.93^{a}	0.60	0.0003
Lactose (%)	2.86	2.94	0.09	0.4330
Other Solids (%)	4.35	4.55	0.09	0.0554
Total Solids (%)	23.24^{b}	21.04^{a}	1.05	0.0439
Mun	30.96	25.98	2.54	0.0593
SCC (*1,000)	2151.7	2864.62	640.04	0.2857
SCCLog	5.74^{a}	6.19^{b}	0.16	0.0051
TPC Log	4.92^{a}	5.45^{b}	0.11	<0.0001
Coliform log	1.3^{a}	1.98^{b}	0.09	<0.0001

[ab] Differences between means are indicated by different alphabetical superscripts (P < 0.05)

Table 4. Percentage of samples distributed across IgG concentrations

IgG (mg/ml)		Samples	(%)
< 50		243	29.38
50 - 80		303	36.64
80 - 100		156	18.86
100 - 120		75	9.07
> 120		50	6.05
	Total	827	100

Table 5. Percentage of samples distributed across total plate counts

TPC (CFU/ml)	Samples	(%)
< 100,000	409	54.8
100,000 - 300,000	90	12.1
300,000 - 500,000	47	6.3
500,00 - 1,000,000	74	9.9
> 1,000,000	126	16.9
Total	746	100

Table 6. Percentage of samples distributed across TPCs by breed

	Unknown[a]		Holstein[b]		Jersey[c]	
TPC (CFU/ml)	Samples	(%)	Samples	(%)	Samples	(%)
< 100,000	11	7.97	239	55.07	59.00	79.73
100,000 - 300,000	34	24.64	48	11.06	8.00	10.81
300,000 - 500,000	23	16.67	23	5.30	1.00	1.35
500,00 - 1,000,000	24	17.39	47	10.83	3.00	4.05
> 1,000,000	46	33.33	77	17.74	3.00	4.05
Total	138	100	434	100	74	100

[a] Unknown breed (includes crossbred) TPC range = 0 - 2,000,000 cfu/ml

[b] Holstein TPC range = 0 - 5,900,000 cfu/ml

[c] Jersey TPC range = 0 - 2,000,000 cfu/ml

Table 7. Percentage of samples distributed across TPCs by storage method prior to feeding

	Fresh[a]		Refrigerated[b]		Frozen[c]	
TPC (CFU/ml)	Samples	(%)	Samples	(%)	Samples	(%)
< 100,000	122	67.03	35	23.03	252	61.17
100,000 - 300,000	21	11.54	17	11.18	52	12.62
300,000 - 500,000	9	4.95	10	6.58	28	6.80
500,00 - 1,000,000	8	4.40	32	21.05	34	8.25
> 1,000,000	22	12.09	58	38.16	46	11.17
Total	182	100	152	100	412	100

[a] TPC range = 0 - 5,900,000 cfu/ml

[b] TPC range = 0 -5,900,000 cfu/ml

[c] TPC range = 0 - 5,900,000cfu/ml

Table 8. Percentage of samples distributed across adequate IgG concentrations and TPCs

Quality		Samples	(%)
> 50 IgG and < 100,000 TPC		294	39.41
> 50 IgG and > 100,000 TPC		233	31.23
< 50 IgG and > 100,000 TPC		104	13.94
< 50 IgG and < 100,000 TPC		115	15.42
	Total	746	100

Table 9. Percentage of samples distributed across adequate IgG concentration and TPC by pooling

	Not pooled		Pooled	
Quality	Samples	(%)	Samples	(%)
> 50 IgG and < 100,000 TPC	272	41.21	22	25.58
> 50 IgG and > 100,000 TPC	201	30.45	32	37.21
< 50 IgG and > 100,000 TPC	81	12.27	23	26.74
< 50 IgG and < 100,000 TPC	106	16.06	9	10.47
Total	660	100	86	100

Table 10. Percentage of samples distributed across adequate IgG concentration and TPCs by breed

		Unknown		Holstein		Jersey	
Quality		Samples	(%)	Samples	(%)	Samples	%
> 50 IgG and < 100,000 TPC		77	32.35	170	39.17	47	63.51
> 50 IgG and > 100,000 TPC		90	37.82	132	30.41	11	14.86
< 50 IgG and >100,000 TPC		37	15.55	63	14.52	4	5.41
< 50 IgG and <100,000 TPC		34	14.29	69	15.90	12	16.22
	Total	238	100	434	100	74	100

Table 11. Percentage of samples with above and below industry recommended adequate IgG concentration and TPC by breed

	Fresh		Refrigerated		Frozen	
Quality	Samples	(%)	Samples	(%)	Samples	%
> 50 IgG and< 100,000 TPC	76	41.99	30	18.52	188	46.65
> 50 IgG and > 100,000 TPC	38	20.99	86	53.09	109	27.05
< 50 IgG and >100,000 TPC	21	11.60	38	23.46	45	11.17
< 50 IgG and <100,000 TPC	46	25.41	8	4.94	61	15.14
Total	181	100	162	100	403	100

CHAPTER SIX

DEVELOPMENT OF THE CAPRYLIC ACID TEST AND COMPARING IT TO THE REFRACTIVE INDEX OF WHOLE SERUM AS A METHOD TO DETERMINE SERUM IgG CONCENTRATION IN NEONATAL CALVES

ABSTRACT

Caprylic acid **(CA)** has been utilized to fractionate colostral immunoglobulins **(IgG)** for further laboratory purification and analysis. Our laboratory has adapted a technique utilizing CA and refractometry to determine the IgG concentration in maternal colostrum. The objective of this study was to develop a rapid, calf-side test to determine serum IgG concentration using CA fractionation followed by refractometry of the IgG-rich supernatant and compare it to refractometry of whole calf serum. Frozen serum samples (n = 200) obtained from 1 d old calves were thawed and treated with three different concentrations of CA and acetic acid **(AcO)**, and then centrifuged for 0, 10 or 20 min. CA test supernatant was analyzed with a digital refractometer (SPER Scientific, model 300034) to determine refractive index **(nD)**. The nD of Ig-rich fraction was compared to total serum IgG concentration determined by radial immunodiffusion **(RID;** Triple J Farms, Bellingham, WA). Whole serum refractive index measurements (nD and brix) were positively correlated with RID-obtained IgG concentration (r = 0.87 and r = 0.86, $P < 0.0001$). Samples treated with either 0.5 ml of 0.06 M AcO and 45µl CA or 1 ml 0.6 M AcO and 60 µl CA and not centrifuged prior to analysis of the supernatant resulted in strong relationship between nD and RID obtained IgG concentration (r = 0.86, n = 41, $P < 0.0001$ and r = 0.80, n = 45, P <

0.0001, respectively). These results suggest that refractometry of whole calf serum provides a strong estimate of IgG concentration that can be used to determine if adequate passive transfer has occurred in 1 d old calves.

Keywords: passive transfer, immunoglobulins, refractometer, calves

INTRODUCTION

Newborn calves are born aggamaglobulinemic, without any measurable circulating IgG or IgM. The newborn calf derives passive immunity by absorbing immunoglobulins **(IgG)** from colostrum **(MC)** provided within the first hours of life (Smith et al., 1964). Passive immunity can be both local (IgG bathing the gut lumen) and humoral (IgG absorbed from the gut into the blood; Lecce, 1984). In the calf, passively acquired immunity is of importance to the health of the calf for the extended period of time until they are capable of making their own antibodies (Smith, 1948).

Calves obtain passive transfer postpartum and are able to absorb large protein molecules through their intestines without prior digestion or alteration of the molecules for a short period postpartum. Nonselective transport occurs by vesicular transport of macromolecules that adhere to the surface membrane or a transported in the fluid-phase compartment of the vesicles (Pácha, 2000). Within the calf, the process of absorption is primarily nonselective, as it has been observed that a wide variety of macromolecules can be absorbed through the gut into the blood (Lecce, 1972). The absorption of macromolecules is divided into three phases (Staley and Bush, 1985): binding of Ig by the microvillus border followed by endocytosis of the binding site and Ig, enlargement of the tubular end piece to form a vacuole, and once the vacuole comes in contact with the cell membrane, the vacuole exocytoses its contents into the lamina propria where it passes into the lymphatics or portal circulation (Fey, 1971; Staley, 1971). Rapid postnatal growth of the intestine results in the replacement of fetal-type enterocytes by adult-type enterocytes (Smith, 1985) leading to gut closure or cessation of macromolecule absorption from gut to blood. Neonates that obtain transfer of antibodies post-partum have short closure times, with cessation of macromolecule transport increasing after 12 h with a mean closure time of 24 h after birth (Stott et al., 1979a; Bush and Staley, 1980). When calves are fed iodine-125 labeled IgG after 36 h, none is observed in the blood, thus suggesting that closure is complete within the first 36 h of life (Devery et al., 1979).

Passive transfer of IgG can then be determined by taking a blood sample from the calf at 24 h of age. Neonatal calves are classified as having failure of passive transfer **(FPT)** if their serum IgG concentration is less than 10 mg/ml at 24 h of age. Radial immunodiffusion **(RID)** is a direct measurement of IgG concentration and is considered to be the gold standard to determine IgG concentration in bovine serum. Unfortunately RID assays require a relatively long incubation time (~24 h) that prevents the identification of FPT calves prior to gut closure. Additionally, high levels of discrepancy between RID kits exist and may be due to inaccuracies in the internal standards (Ameri and Wilkerson, 2008). Refractometers, digital or optical, have been utilized to measure the total protein **(TP)** content in MC and calf serum (Calloway, 2002; Bielmann, 2010). Protein solutions refract light, and refractometers use this property to measure TP in a solution (Chavatte, et al., 1998). In the neonatal calf, IgG constitutes a large proportion of the protein in serum. This allows the measurement of TP to provide an estimation of serum IgG concentration.

While the TP of serum is a helpful tool, knowing the actual IgG concentration would be of greater benefit. Short chain fatty acids have long been recognized to be powerful plasma protein precipitants (Chanutin and Churnish, 1960). Caprylic acid **(CA)** has been utilized to precipitate non-IgG proteins from a solution, leaving an IgG-rich supernatant (Bernard et al., 1995). This technique has been adopted to determine the IgG content of mammary secretions from non-lactating dairy cattle (Guidry and O'Brien, 1996) and for the purification of serum for therapeutic uses in humans (Bergmann-Leitner et al., 2008; Parkkinen et al., 2006: Peroso et al., 1990) and horses (Mpandi et al., 2007). Our laboratory has adapted the use of CA to precipitate non-IgG proteins in MC leaving an IgG-rich supernatant that can be measured using a refractometer. The refractive index **(nD)** of the supernatant has a strong relationship to the RID determined IgG concentration (r = 0.96, $P < 0.0001$; Morrill et al., unpublished).

The objectives of this study were to adapt the CA test to neonatal calf serum and compare the CA test and refractometry of whole serum in ability to rapidly and accurately assess IgG concentration.

MATERIALS & METHODS

Frozen serum samples (n = 200) from 1 d old Holstein calves on a California calf ranchwere provided by APC, Inc. (Ankeny, IA). Samples were allowed to thaw at room temperature prior to analysis.

Serum Sample Analysis

Refractometer reading – whole serum

A drop of whole serum (~50 µl) was placed on a refractometer prism (SPER SCIENTIFIC model 300034; Scottsdale, AZ) and a %Brix and nD reading was recorded for each sample. The digital refractometer determines the nD of the liquid being analyzed by shining a light through the sample in the well, measuring the index of refraction and presenting the reading in nD or %Brix units on a digital scale. The nD values is the refractive index of a solution, measured at the wavelength of the sodium D line (589.3 nm) at 20 °C. The brix value can be obtained from the polynomial fit to the ICUMSA (2009) table: brix = (((((11758.74*nD -88885.21)*nD + 270177.93)*nD - 413145.80)*nD + 318417.95)*nD - 99127.4536).

Caprylic acid test analysis

One ml of bovine calf serum was added to a tube containing one of three acid treatments (Table 1) and mixed for 10 sec and allowed to incubate for 60 sec. Samples were then centrifuged for 0, 10 or 20 min prior to analysis of the supernatant by digital refractometry.

Statistical Analysis

The PROC CORR procedure of SAS (SAS 9.2, SAS Institute Inc., Cary, NC) was utilized to determine the relationship between RID obtained IgG concentration of bovine serum and the brix or nD value obtained by refractometry for each treatment.

RESULTS

Two hundred serum samples were analyzed, 15 samples had IgG concentrations less than 3.43 mg/ml (lowest internal standard) and were not included in the analysis of the relationship between nD and RID determined IgG concentration. The mean IgG concentration of the remaining 185 serum samples was 18.98 mg/ml (SD = 9.68) with a range of 3.45 to 47.01 mg/ml. A total of 150 of the samples had IgG concentrations greater than 10 mg/ml indicating adequate passive transfer had occurred, while 50 samples had IgG concentrations less than 10 mg/ml indicating FPT.

Correlations

Whole serum nD was positively correlated with RID obtained IgG concentration ($r = 0.86$, $P \leq 0.00$; Figure 1). Correlation coefficients for the CA test treatment refractometry values compared to RID obtained serum IgG concentrations were positive, but had weaker relationships compared to that observed between whole serum nD and RID obtained IgG concentrations (Table 2). The correlation between TRT B samples that were not centrifuged and RID obtained IgG concentration ($r = 0.80$, $P \leq 0.001$) provided the strongest relationship for the CA test variables. This suggests that refractometry of whole serum provides the best estimate of IgG concentration.

Accuracy

Using the regression equation IgG = 5919.1*nD - 7946.1, over 76% of the samples were estimated within 5 mg/ml of the RID determined IgG concentration (Table 3). Serum samples that were overestimated had similar LSMEAN RID concentrations (14.83, 14.42 and 10.55 mg/ml, respectively) and lower LSMEAN RID concentration ($P < 0.05$) as compared to samples that were underestimated. The RID determined IgG concentration of samples that were underestimated increased ($P < 0.05$) as the difference between actual and estimated IgG increased (21.57, 28.91 and 34.67 mg/ml, respectively).

DISCUSSION

The percentage of calves utilized in this study that had FPT is slightly greater than the 19.2% estimated by Beam et al. (2009) utilizing data in the 2007 NAHMS report and identical to that reported in Ontario dairy calves (Wallace et al., 2006). In the calf, passively acquired immunity is of importance to the health of the calf for the extended period until they are capable of making their own antibodies (Smith, 1948). Failure of passive transfer introduces the risk of increased mortality (Tyler et al., 1999), and morbidity due to an increased susceptibility to pathogens and subsequent disease (Boyd, 1972; McGuire, 1976).

Bielmann and Leslie (unpublished) utilized a refractometer to evaluate TP in bovine calf serum at 24 to 36 h of age and again at 11 to 14 d of age. They observed a nearly identical relationship between TP and RID obtained IgG concentration ($R^2 = 0.7349$) as we observed between nD of whole serum and RID obtained IgG concentration.

There are many advantages of refractometry of whole serum to determine IgG over currently available methods. The current industry gold standard, RID, has a long incubation time (18 to 24 h) preventing identification of FPT calves prior to gut closure. Refractometry takes only the time necessary to obtain a blood sample, allowing it to sit for the serum to separate and less than 15 sec for the refractometer to produce a digital reading. Refractometry does not require internal standards that may introduce error in determining the IgG concentration of serum (Ameri and Wilkerson, 2008). Digital refractometers are calibrated with distilled water that is readily available. A second strength of refractometers is that the accuracy is minimally impacted by temperature (Calloway et al., 2002). Digital refractometers often contain a temperature compensating device, thus reducing the confounding factor of temperature that can impact the accuracy of other methods to determine IgG concentration in serum. The sodium sulfate precipitation test provides accurate results when the test is carried out at a consistent temperature (4^oC or 38^oC) but provides inconsistent results when the temperature fluxuates ($28 – 30^o$; Aba-Adulugba et al., 1989). The simplicity of the refractometer to determine the IgG concentration of serum as compared to RID, ELISA, zinc sulfate turbidity test, sodium sulfate precipitation test and glutaraldehyde coagulation test is a great advantage and will easily allow for the on-farm adaptation.

The primary disadvantage of refractometry to determine FPT in calves is that it currently requires the use of serum. Very few dairy farms own a centrifuge, however, data out of Canada reports that serum collected from blood tubes allowed to sit and clot had a total protein content (as determined by refractometer) that was highly correlated ($R^2 = 0.95$, n = 234) to the total protein content of a duplicate sample that was centrifuged prior to serum collection (Wallace et al., 2006). This suggests that producers could take a blood sample from calves, let it sit and use a transfer pipette to remove a small amount of the serum for analysis by refractometer.

Currently only 2.1% of U.S. dairy operations routinely measure passive transfer status of calves (NAHMS, 2007). The goal of any calf monitoring program is not to predict the health fate of each calf, but to monitor the success of passive transfer on an individual farm and provide additional support to FPT calves. If producers have access to simple tools that rapidly and accurately estimate IgG concentration, FPT calves and calves at risk of FPT could be identified and provided additional MC, or preventative care.

CONCLUSION

The objectives of this study were to determine if the CA could be adapted to serum and compare this method to refractometry of whole serum as a method to evaluate IgG concentration of neonatal calves. Adding 1 ml of serum to a tube containing 1 ml 0.06 M acetic acid, 60 µl caprylic acid, shaking the sample for 10 sec, allowing the sample to incubate for 1 min prior to analysis of supernatant resulted in a strong correlation between nD and RID. However, a stronger relationship exists between the nD of calf serum, that has not gone through the CA test, and IgG concentration as determined by RID. This study concludes that the refractive index of whole serum provides a rapid and accurate estimate of serum IgG concentration.

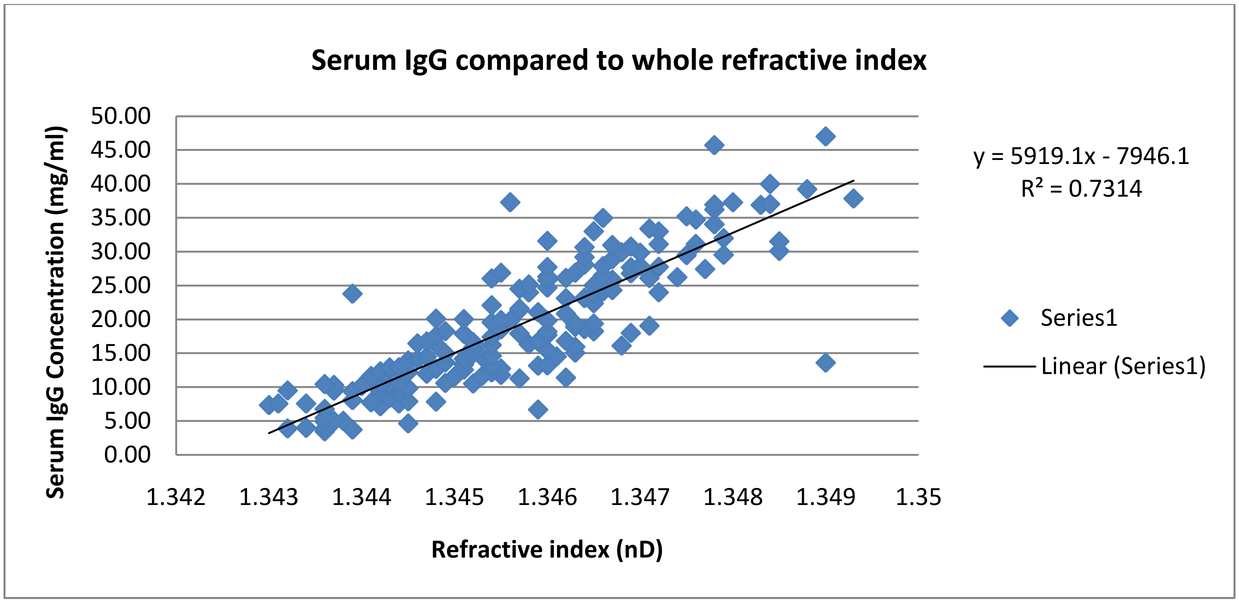


Figure 1. Serum IgG concentration as determined by RID compared to the refractive index (nD) of whole calf serum. n = 185; r = 0.855

Table 1. Caprylic acid and acetic acid concentrations evaluated to determine the relationship between nD and RID obtained IgG concentration.

	TRT A	TRT B	TRT C
serum (ml)	1.0	1.0	1.0
0.6 M acetic acid (ml)	0.5	1.0	1.5
Caprylic acid (µl)	45	60	75

Table 2. Correlation coefficients between RID determined serum IgG concentration and brix or nD values.

		RID*Brix		RID*nD	
	n	r	p - value	r	p - value
Treatment[1]					
Whole serum	185	0.87	<0.0001	0.86	<0.0001
TRT A - NC	41	0.61	<0.0001	0.61	<0.0001
TRT A -10	40	0.72	<0.0001	0.72	<0.0001
TRT A - 20	39	0.76	<0.0001	0.77	<0.0001
TRT B - NC	45	0.77	<0.0001	0.8	<0.0001
TRT B - 10	41	0.78	<0.0001	0.77	<0.0001
TRT B - 20	40	0.72	<0.0001	0.52	<0.0001
TRT C - NC	53	0.56	<0.0001	0.59	<0.0001
TRT C - 10	51	0.69	<0.0001	0.69	<0.0001
TRT C - 20	35	0.35	<0.0001	0.75	<0.0001

[1] Treatment number followed by the number of minutes allowed to sit prior to centrifugation, where NC = no centrifuge, TRT A = 0.5 ml acetic acid & 45 µ caprylic acid, TRT B = 1 ml acetic acid & 60 µl caprylic acid, TRT C = 1.5 ml acetic acid & 75 µl caprylic acid.

Table 3. Percentage of samples estimated within 5, 10 and greater than 10 mg/ml of the actual IgG concentration

	Underestimated		Overestimated	
	n	%	n	%
Actual - estimated IgG[1]				
< 5 mg/ml	69	37.30	72	38.92
5 - 10 mg/ml	16	8.65	20	10.81
> 10 mg/ml	5	2.70	3	1.62

[1] Using the regression equation: IgG = 5919.1 * nD - 7946.1

CHAPTER SEVEN

EXPLORING AN EX-VIVO TECHNIQUE TO DETERMINED MACROMOLECULE ABSORPTION IN THE NEONATAL RUMINANT

ABSTRACT

Newborn calves require maternal antibodies from maternal colostrum **(MC)** or colostrum replacer **(CR)** to obtain adequate passive transfer. Colostrum replacers have been created to provide nutrients and antibodies needed, when high quality MC is not readily available. The objective of this study was to evaulate the use of the Ussing chamber as a method to evaluate immunoglobulin **(IgG)** transport across the neonatal calf small intestine. Five Holstein bull calves were euthanized by captive bolt within 20 min post-partum. The small intestine was removed and samples were obtained from the mid-jejunum. Eight intestinal samples from each calf were obtained, transported to the laboratory, placed in the Ussing chamber, and exposed to one of four CR treatments (A = 25, B = 50, C = 75 or D = 100 mg IgG/ml) on the luminal side and 1 of 2 buffer solutions (Krebs buffer or fetal calf serum) on the serosal side. Mean IgG concentration did not change across time points (0 – 150 min) on the serosal side, regardless of CR or buffer treatment. Mean IgG concentrations on the luminal side decreased ($P < 0.01$) from 0 to 150 min for treatments A and B, but not for C and D when the Krebs buffer was present on serosal side. Mean IgG concentration on the luminal side did not change across treatments when fetal calf serum was present on

serosal side. These results suggest that the Ussing chamber is not a valid method to evaluate IgG transport mechanisms in bovine neonatal intestinal samples.

Keywords: Calves, colostrum, transport, IgG

INTRODUCTION

Newborn animals of many species rely on maternal colostrum **(MC)** to provide them with the nutrients needed to sustain life. Immunoglobulins **(Ig**) cannot cross the placental structure of cattle and calves are born aggamaglobulinemic with no measurable circulating IgG or IgM. High quality MC fed within the first hours of life provides the calf with sufficient amounts of IgG to provide passive immunity for the first 30 to 90 days of life (Guy et al., 1994) until the immune system of the calf is better equipped to respond to pathogens on its own.

In utero the fetal calf obtains nutrients from the placenta. During the third trimester, expression of transporters allows the fetus to obtain carbohydrates, amino acids (AA) and proteins from swallowed amniotic fluid (Buchmiller et al., 1992). Birth represents one of the greatest physiological adaptations; the calf must adjust from obtaining all nutrients from the placenta to intestinal absorption of nutrients received in MC and milk. At birth the neonatal small intestine has the capability to absorb nutrients along the whole crypt-villus axis, as fetal-type enterocytes are replaced by adult-type enterocytes, nutrient uptake will be shifted to the upper part of the villus (Pácha et al., 1991).

Absorption of colostral IgG during the first 24 h of life is nonspecific, thus allowing IgG to be taken up by fetal-type enterocytes of the small intestine and transferred into the lymph spaces, and then into the blood circulation. Closure of the intestinal permeability to colostral IgG in the calf occurs spontaneously with a progressively increasing rate after 12 h postpartum, with mean closure near 24 h (Stott, 1979). Gut closure appears to be an energy-dependent process, because both hypoxia at birth (Tyler and Ramsey, 1991) and insulin-mediated hypoglycemia (Tyler and Ramsey, 1993) delay gut closure. With increasing age there are progressively fewer intestinal epithelial cells capable of pinocytotic activity, colostral uptake and transmission of colostral constituents into circulation (Stott, 1979).

Current research on factors affecting passive transfer of IgG from MC or colostrum replacers **(CR)** relies on *in vivo* studies that require large numbers of calves. These studies rely on the researcher preventing the calf from suckling, obtaining a blood sample immediately after birth, and feeding MC or CR at the same time to all the calves on the study. It also involves feeding and bleeding each calf at precisely specified time points for at least the first 24 h of life. These studies are expensive, time consuming and have the potential to introduce other variables (season, dystocia, climate, etc...) that may have confounding effects on IgG absorption.

The development of a cost effective, *ex-vivo* procedure that can easily be used to determine macromolecule absorption in neonatal ruminants would be beneficial to researchers and the dairy industry. This would allow researchers to test an additive or new formulation of colostral supplements, or CR prior to a field study, thus minimizing the need for lengthy trials that may show no benefit. The mechanisms of nutrient transport have, in part, been determined through the use of either *in-vitro* or *ex-vivo* techniques. These techniques have included intestinal rings, everted intestinal sacs, ligated intestinal segments, isolated mucosal cells, isolated brush border membrane vesicles, basolateral membrane vesicles and intestinal segments mounted in Ussing chambers. The uses of the first four techniques have limitations in that cellular metabolism is occurring and that the researcher cannot regulate the intracellular substrate and electrolyte concentrations (Wilson and Webb, 1989). The use of isolated membrane vesicles has overcome some of these problems, and give researchers the ability to characterize nutrient transport systems at both the luminal and serosal membrane side of the mucosal lining (Wilson and Webb, 1989).

Wilson and Wiseman (1954) developed an *ex vitro* method in which a preparation of isolated small intestine from the rat or golden hamster was everted to measure respiration and glycolysis during periods of active transport of substances across the intestinal wall. The use of everted sections of intestinal segments has been used to determine in vitro absorption of amino acids in sheep (Phillips et al., 1976), γ-globulin in piglets (Lecce, 1966) and mice (Lecce, 1972) and the transference of other substances from the mucosal to the serosal surface (Wilson and Wiseman, 1954). Lecce (1965) modified the *in vitro* method and was able to demonstrate pinocytosis in mammalian intestinal cells.

Isolated intestinal brush border membrane vesicles have the advantage over isolated small intestine samples in that transport of a substrate across the intestinal brush border membrane can be studied without interference of intracellular metabolism (Hopfer, 1977). Under appropriate homogenization conditions, the isolated brush border membranes form vesicles so that the membrane separates two aqueous phases, the medium and the intravesicular space (Hopfer, 1977). By measuring solute flux in or out of these vesicles, the transport properties of the membrane can be determined.

Wilson and Webb (1989) demonstrated that brush border and basolateral membrane fractions were osmotically active and suitable models for evaluating nutrient transport properties of the bovine intestinal enterocytes. Some of the information that has been obtained with the brush border membrane vesicles include 1) mechanisms of solute translocation across both plasma membranes, 2) coupling of Na^+ and non-electrolyte transport in the brush border membrane, 3) kinetics of non-electrolyte transport under controlled *cis* and *trans* conditions, and 4) analysis of transport changes associated with different physiological or pathological stress (Hopfer, 1977). The use of purified brush border membranes vesicles from the calf jejunum have also been utilized to demonstrate an active Na^+ gradient-dependent nucleobase transport (Theisinger et al., 2003).

Ussing chambers have been used as an *ex-vivo* model for intestinal transport. Intestinal tissue is collected and mounted between two chambers containing reservoir buffer (the luminal and serosal chambers) permitting the study of absorption of compounds across the mounted tissue. Ussing chambers have been used to investigate nutrient absorption across the gut epithelial tissue for various animal species (Awati et al., 2009). After a review of the literature, however, it becomes apparent that there is limited information on the use of Ussing chambers to determine intestinal transport of nutrient in young animals, let alone neonatal animals.

Ussing chambers provide the benefit of reduced variability among samples, and a more controlled environment as compared to the everted intestinal sac technique. The primary objective of this research was to determine if the Ussing chamber is a valid model to determined macromolecule transport across the neonatal ruminant small intestine.

MATERIALS & METHODS

All procedures were approved by the Iowa State University Animal Care and Use Committee prior to the start of the project (IACUC # 3-10-6912-B).

Animals

Six Holstein bull calves were removed from their dam immediately after birth and euthanized by captive bolt within 20 min of birth. One Holstein bull calf was fed a CR base containing no IgG immediately after birth and euthanized by captive bolt 1 h after birth. After stunning with the captive bolt calves were exsanguinated via severance of the jugular vein and carotid arteries, and the abdomen was opened by a midline incision. The entire small intestine was isolated and washed with a Krebs buffer solution. The small intestine was removed by cutting the proximal end of the duodenum and the distal end of the ileum. The intestine was measured end to end and cut at 40% of the total length to obtain mid-jejunal samples (20 to 30 cm). The sample was placed in chilled Krebs buffer for transport back to the laboratory (less than 20 min) under constant aeration until clamped in the Ussing chamber.

Ussing Chamber procedure

Once in laboratory the intestinal tissue was stripped of outer muscle layers and immediately mounted in Ussing Chambers (DVC 1000 World Precision Instruments). Each segment was bathed on its mucosal and serosal surfaces (opening area 1.0 cm^2) with 8 ml Krebs solution and gassed with 95% O_2-5% CO_2 mixture. After a 10 min period to allow the tissues to stabilize, the Krebs buffer on the mucosal side was removed and replaced with the respective CR treatment. This was repeated on seven different days with a total of seven neonatal calves.

Treatments – Trial #1

Colostrum replacer was mixed to create four different IgG concentrations; 100 mg IgG/ml, 75 mg IgG/ml, 50 mg IgG/ml, or 25 mg IgG/ml. The CR treatment (4 ml) was added to the chamber exposed to the mucosal side of the tissue sample. The chamber exposed to the serosal side of the tissue had 4 ml of Krebs buffer or fetal calf serum added to it. Samples from both chambers (400 µl) were taken at 0, 30, 60, 90, 120 and 150 minutes to be analyzed for IgG concentration.

Treatments – Trial #2

Three treatments were compared in trial # 2: CR at 2 IgG concentrations or MC. Colostrum replacer was mixed to create 2 different IgG concentrations75 mg IgG/ml **(CR-A)** or 25 mg IgG/ml **(CR-B)**. Maternal colostrum was collected prior to the trial and contained 55 mg IgG/ml. IgG. The treatment (4 ml) was added to the chamber exposed to the mucosal side of the tissue sample. Four ml of Krebs buffer was added to the chamber exposed to the serosal side of the tissue. Samples were obtained from both chambers (400 µl) at 0, 30, 60, 90, and 120 min to be analyzed for IgG concentration.

IgG analysis

Samples were analyzed for IgG concentration by enzyme-linked immunosorbant assay. Sheep anti-bovine IgG_1 (Bethyl Laboratories) was used as the coating antibody, Bovine reference serum (12 mg/ml IgG_1; Bethyl Laboratories) was used as the calibrator and

Sheep anti-bovine IgG_1-HRP conjugate (Bethyl Laboratories) was used as the detection antibody.

Statistical analysis

All data are presented in means $\pm$ SE. Serosal and mucosal IgG data from trial #1 were analyzed using the PROC MIXED procedure in SAS (Version 9.2, SAS Institute) according to the model:

$$Y = \mu + B_i + C_j + T_k + e_{ijk,}$$

where Y = the dependent variable; μ = the overall mean; B_i = the fixed effect of the ith buffer (i = krebs, FCS); C_j = the fixed effect of the jth colostral IgG concentration (j = 25, 50, 75, 100); T_k = the fixed effect of the kth minute (k = 0,…150); and e_{ijk} = the residual error.

Serosal and mucosal IgG data for trial #2 were analyzed using the MIXED procedure of SAS (Version 9.2, SAS Institute) according to the following model:

$$Y = \mu + C_i + T_j + F_k\ e_{ijk,}$$

where Y = the dependent variable; μ = the overall mean; C_j = the fixed effect of the ith colostral treatment (i = CR-25, CR-75, MC-55); T_j = the fixed effect of the jth minute (j = 0,…120); F = the fixed effect of the kth feeding treatment (k = yes, no); and e_{ijk} = the residual error.

For all models and analyses, degrees of freedom were calculated using the Satterthwaite option of the MIXED procedure of SAS (SAS Institute, Inc). Least square means were determined for each treatment. The PDIFF option in SAS (SAS Insitute, Inc.)

was used to seperate least square means among treatments; significance was declared at $P \leq 0.05$.

RESULTS

Trial # 1

Actual treatment IgG concentrations for CR treatments A, B, C and D as determined by ELISA were 67.54, 63.68, 52.53 and 31.88 mg/ml for trial #1. The IgG concentration of treatments A and B were not different; however IgG concentrations of the other treatments were different ($P < 0.001$). Mean IgG concentration did not change across time points on the serosal side of the tissue, regardless of CR treatment or buffer treatment (Fig. 1 & 2). Mean IgG concentrations on the luminal side of the tissue decreased ($p < 0.01$) from 0 to 150 min for treatment A and B, but not for C or D when the Krebs buffer was present on serosal side of the tissue (Fig 3). Mean IgG concentration on the luminal side of the tissue did not change across treatments when fetal calf serum was present on serosal side of the tissue (Fig. 4).

Trial #2

The IgG concentration of the MC used in trial #2 was 54.98 mg/ml. The CR utilized in trial #2 had mean IgG concentrations of 55.06 and 25.99 mg/ml. There was a difference ($P \leq 0.05$) in the IgG concentration of CR-A between the fed and un-fed calf (60.71 and 49.41 mg/ml). The IgG concentration between CR-A and CR-B were different ($P \leq 0.05$) however the MC IgG concentration was not different from CR-A and CR-B. The IgG concentration on the serosal side of the tissue did not change across time-points, regardless of colostrum treatment. The IgG concentration on the mucosal side of the tissue did not change across time-points for treatments CR-A and CR-B (Figure 5). There was an increase in IgG concentration in the hamber on the mucosal side of the tissue from 0 to 120 min for the MC treatment.

DISCUSSION

The postnatal ontogeny of nutrient transporters reflects the need to absorb increasing quantities of nutrients that are required for growth and metabolism (Pácha, 2000). The regulation of intestinal transport depends on the genetic endowment, intrinsic properties and biological clock of enterocytes, systemic and local hormones, growth factors, neurotransmitters and complex interactions between enteric nerves in the submucosa and immune cells in lamina propria as well as environmental factors (Pácha, 2000; Lebenthal and Lebenthal, 1999). Ion transport by the intestinal epithelium is modulated by the enteric nervous system, and stimulation of intestinal intrinsic nerves increase bicarbonate and chloride secretion in rats (Carey, 1989). Kurz and Willet (1991) reported that gamma-glutamyl transferase, alkaline phosphatase and glutamic ocaloacetic transaminase activity did not occur in calves until after the first feeding of MC. In newborn infants, gastronintestinal secretory and absorptive function is influence by both the amount of effective hormones reaching the target cells, but also the number and affinity of receptors on its surface (Lebenthal and Lebenthal, 1999).

A great deal of research has been conducted to determine what impacts gut closure and improves AEA of IgG, however there is limited data on what stimulates fetal-type enterocytes to internalize IgG and exocytose it to the lamina propria. Neonatal calves in this study were euthanized immediately after birth and prior to ingestion of CR. This introduced two factors that would not normally be present when MC or CR would be fed to the calf. The calf was never sentient or conscious and the potential effect of suckling and dietary effects on stimulation of hormones such as cholecystokinin **(CCK)**, gastrin and secretin, and neurotransmitters were eliminated (Lebenthal and Lebenthal, 1999). Both of these factors may be necessary for IgG uptake to occur within the neonatal calf intestinal enterocytes.

Sentience is a state of awareness or consciousness and requires a nervous system that is functionally capable of cognition (Mellor, 2010). Calves are classified as neurologically mature at birth, but may not exhibit consciousness for a few hours after birth, this leads to a continuation of neuro-inhibition and lower nuero-activation (Mellor, 2010). Additionally during parturition, there are O_2 sparing mechanisms that lead to the fetal motor systems becoming inactive during the delivery process, as well as protecting the brain from hypoxia after birth (Mellor, 2010). Synaptic activity due to neurotransmitter release is the cellular level mechanism that makes cognition possible while actions potential due to in flux is the cellular level mechanism that makes consciousness possible (Cook, 2008; Levine, 2001). The lack of consciousness in the calves used in this study would prevent signal propagation within the neurons which would be necessary for permeability of the neuronal membrane allowing a flux of charged ions into the cellular interior (Cook, 2008) thus potentially eliminating neurotransmitter signals necessary to stimulate fetal-type enterocyte uptake of IgG.

Gut regulatory peptides such as gastrin and CCK increase at the end of gestation and after birth (Guilloteau et al., 2009) and have been implicated in the regulation of GIT development in the fetus (Guilloteau et al., 1998) and during postnatal development and weaning (Guilloteau et al., 1992a). Blocking of mucosal CCK_1 receptor by pharmacological antagonists significantly delays the disappearance of vacuolated fetal-type enterocytes that are a characteristic feature of the immature intestinal tract of calves and lambs (Guilloteau et al., 2008; Biernat et al., 1999). Fetal-type enterocytes lack lysosomal activity during the first day of life, and it has been suggested that this helps to transfer colostral bioactive substances in an intact form into circulation (Baintner, 2002; Brown and Moon, 1979). The functionality and life-span of fetal-type enterocytes is dependent on a number of hormonal factors regulating maturation of gut epithelium (Biernat et al., 1999; Guilloteau et al., 1992a). Feeding MC leads to increases in plama gastrin, CCK, and pancreatic polypeptide within 1 h after feeding and significant increases in plasma gastrin, CCK, secretin, and vasoactive intestinal peptide 10 h after feeding as compared to fasted calves (Guilloteau et al., 1992b). In neonatal piglets, insulin is involved in the regulation of both macromolecule transfer from the gut lumen into circulation and gut closure by initiating the synthesis of membrane structural proteins in the enterocytes (Svendsen et al., 1986). These studies support the idea that MC feeding modulates the release of several regulatory peptides shortly after birth and first feeding that impact IgG uptake and transport by fetal-type enterocytes. Based on our data, in which no transport of IgG was observed, it is possibles that the lack of endogenous hormones inhibited the fetal-type enterocytes ability to take up macromolecules.

While the lack of IgG transport across the epithelial cells makes the Ussing chamber a poor method to determine macromolecule transport in neonatal small intestinal samples, it supports previous research that IgG is not transported into the laminia propria by paracellular transport in newborn calves (Jochins et al., 1994a) or newborn rats (Jochins et al., 1994b). If paracellular transport occurred there would have been IgG disappearance from the mucosal chamber, which was not observed in this study.

CONCLUSION

No changes in IgG concentration from the mucosal side of the tissue were observed in this study. These data suggest that the Ussing chamber is not a valid model to determine IgG transport in the neonatal ruminant small intestine. This data suggests that removal of small intestinal tissue from in-vivo exposure to endogenous hormones and neural signals may inhibit the ability of fetal-type enterocytes to actively transport macromolecules such as IgG.

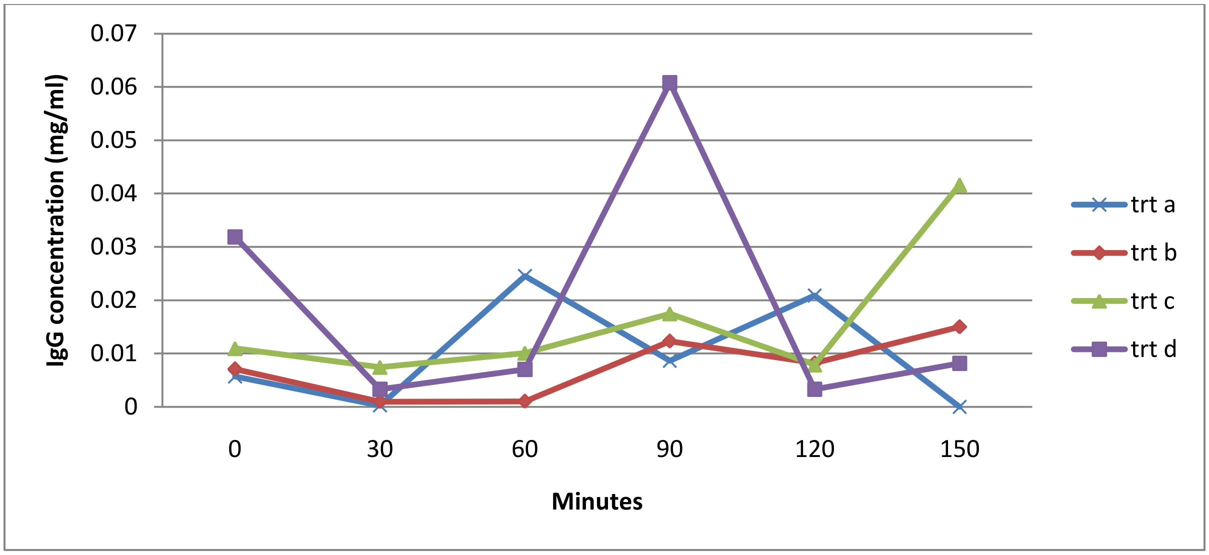


Figure 1. IgG concentration change in krebs buffer on the serosal side of small intestinal sections in an Ussing chamber (n = 5 intestinal samples/trt).

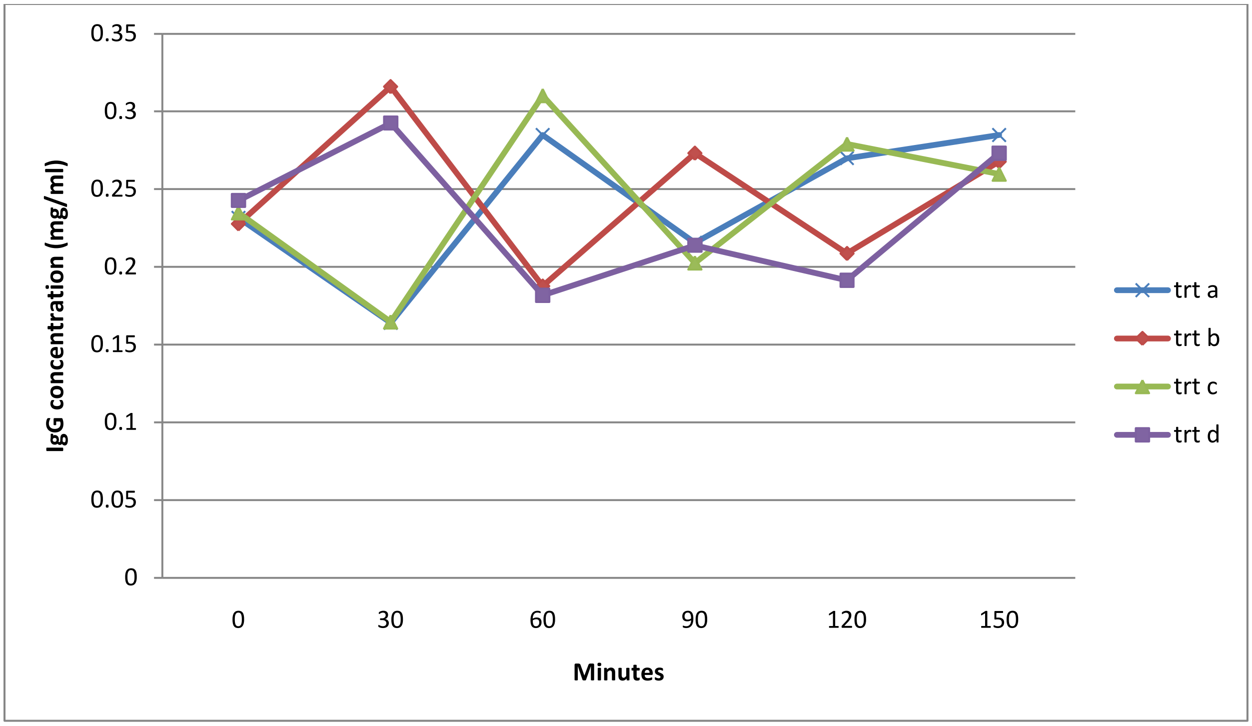


Figure 2. IgG concentration change over time in fetal calf serum on the serosal side of small intestinal tissue mounted in an Ussing chamber (n = 5 intestinal samples/treatment).

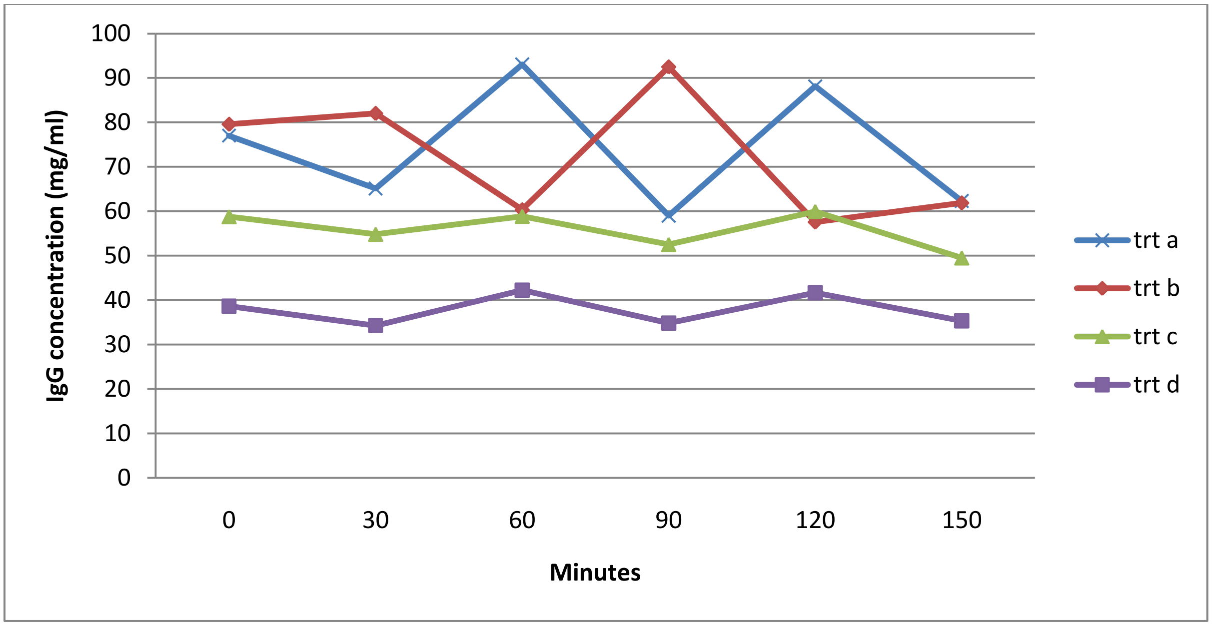


Figure 3. IgG concentration change in colostrum replacer on the luminal side of small intestinal tissuemounted in an Ussing chamber with Krebs buffer on the serosal side (n = 5 intestinal samples/treatment).

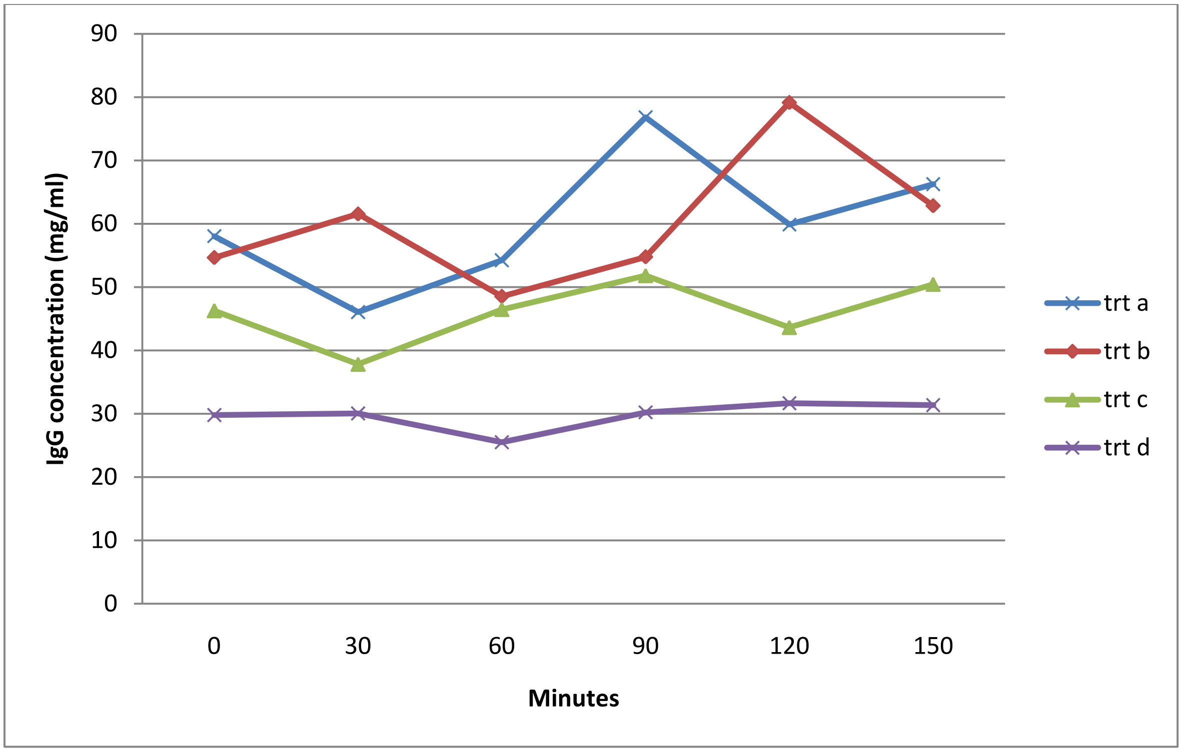


Figure 4. IgG concentration changes in CR on the luminal side of small intestinal tissue mounted in an Ussing chamber with fetal calf serum as the buffer on the serosal side (n = 5 intestinal samples/trt).

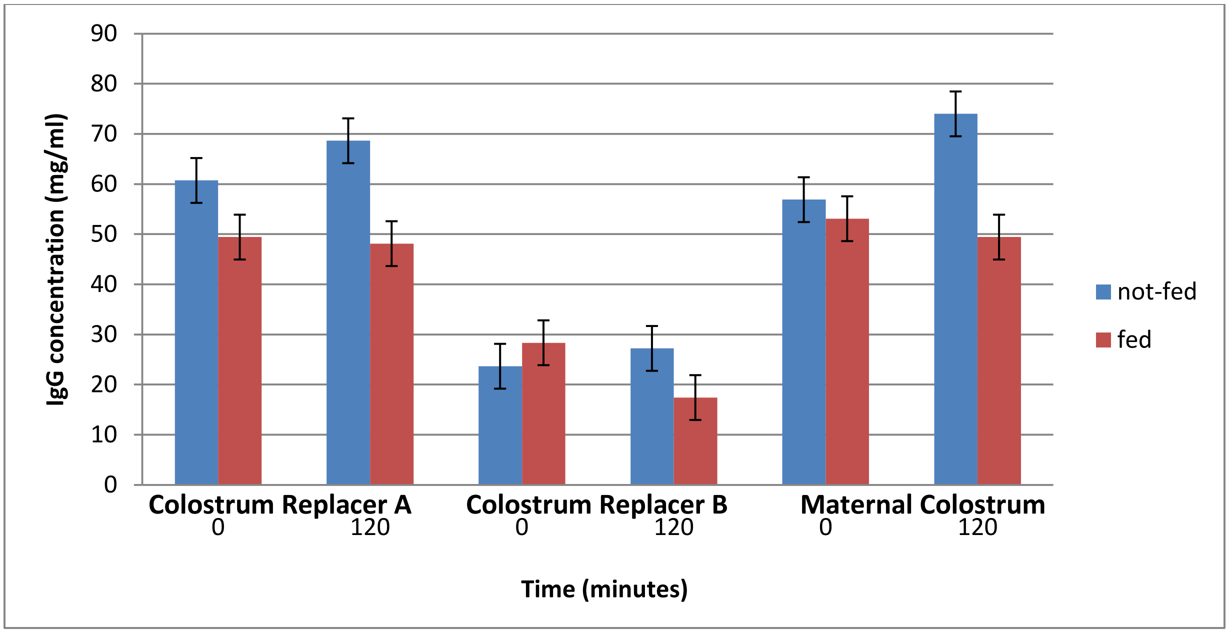


Figure 5. IgG concentration changes on the mucosal side of samll intestinal tissue mounted in an Ussing chamber when the calf was fasted or fed a colostrum replacer base pior to euthanasia. SE = 4.47

CHAPTER EIGHT

GENERAL CONCLUSIONS

The research presented in this dissertation represents a continuing effort to improve methods to determined colostral and serum IgG concentration as well as to better understand what impacts colostrum quality and macromolecule uptake in the small intestine of the neonatal calf. The findings from the studies described in this dissertation support the need for improvements in colostrum management practices on U.S. dairy farms as well as provide details on methods to rapidly and accurately determine colostral IgG content.

Chapter two described a modification of a technique to precipitate non-IgG proteins from non-lactating mammary secretions to adapt it for use with bovine maternal colostrum. This study determined that it was possible to simplify a laboratory based procedure and rapidly assess maternal colostrum IgG concentration with caprylic acid precipitation and refractometry.

Chapter three details the on-farm evaluation of the caprylic acid test and refractometry of whole colostrum for evaluation of colostral IgG concentration. Over the course of five months, 67 dairy farms across the U.S. were visited and maternal colostrum samples were collected. The IgG concentrations of samples were evaluated using the caprylic acid test, refractometry of whole colostrum and radial immunodiffusion. This study concluded that refractometry of whole colostrum provides the greatest relationship between refractive index and actual IgG concentration.

Chapter four focuses on the impact of storage method of colostrum and the number of freeze-thaw cycles on the accuracy of the caprylic acid test and refractometry of whole colostrum. This study concluded that the caprylic acid test provided the most accurate results when colostrum samples were analyzed fresh. Refrigeration, freezing and multiple freeze-thaw cycles had a negative impact on the accuracy of both the caprylic acid test and refractometry of whole colostrum to estimate IgG concentration.

In addition to IgG, colostrum provides the neonate with nutrients necessary for life, and may also be a potential source of pathogenic bacteria. The objective of chapter five was to evaluate the nutrient, bacterial and IgG content of colostrum available on U.S. dairy farms. A wide range in IgG, bacterial and nutrient content was observed in the sample set. Nearly 30% of the colostrum samples fell below the industry recommended value for IgG concentration while nearly 50% of samples did not meet the industry recommended bacterial cut-point. Combined, only 30% of colostrum available on U.S. dairy farms met both industry recommendations for IgG and bacterial contamination. Data presented in this study suggest room for improvement in colostrum management programs on U.S. dairy farms, and also raises awareness on the number of calves that are potentially being put at risk for failure of passive transfer.

Providing producers with tools to evaluate colostrum management and identify calves with failure of passive transfer will increase overall profitability. Chapter six focuses on the validation of the caprylic acid test on serum to identify failure of passive transfer in neonatal calves. This study observed that refractometry of neonatal calf serum provides the strongest estimate of IgG concentration.

Chapter seven explored the possibility of adapting the Ussing chamber as a method to evaluate macromolecule transport across neonatal intestinal segements. Minimal disappearance of IgG from the mucosal chamber was observed. It was concluded that Ussing chambers are not a valid method to determine macromolecule transfor in neonatal small intestine segments. Numerous changes are occurring within the calf during the first hours of life and it is possible that neural and hormonal changes that are not present in an *ex-vivo* procedure are necessary to modulate the uptake and transport of colostral derived IgG by fetal-type enterocytes.

FUTURE RESEARCH

The research presented in this dissertation helps to answer questions regarding colostrum quality, composition and methods to evaluate IgG concentration and transport in the neonatal small intestine. The majority of the colostrum samples utilized in these studies were collected from Holstein cattle, an additional study should be conducted to determine if there is an impact of breed when fresh samples are collected and analyzed by the caprylic acid test and refractometry. Additional questions regarding the adaptation of the caprylic acid test to other species for colostral analysis and to neonatal serum of other species, especially foals, to evaluate if adequate passive transfer transfer has occurred still need to be answered. It would be beneficial to evaluate the impact of bacterial contamination, storage method and number of freeze/thaw cycles of colostrum on the accuracy of the radial immunodiffusion assay. More research is needed on the mechanisms regulating enterocyte uptake of macromolecules during the first hours of life.

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